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The functions of Bcl-2 family proteins in determining the life and death of cells are conserved during evolution. In addition to regulating programmed cell death in mammals, Bcl-2 family proteins can also induce or prevent cell death in the unicellular yeast; overexpression of Bax causes lethality in both *S. cerevisiae* and *S. pombe*, and co-expression of either Bcl-2 or Bcl-XL can protect yeast against Bax-caused cell death. On the basis of these observations, we have developed a functional screen and have identified two human genes, BI-1 and BI-2, which inhibit Bax-induced cell death in yeast. Over the past year, we further characterized these two genes and found that both BI-1 and BI-2 are also involved in regulating apoptosis in mammalian cells. Our characterization of these two genes are reported here. Furthermore, we also undertook a yeast genetic screen isolating yeast mutants that are resistant to Bax-mediated lethality. This genetic screen and subsequent complementation cloning have led to the identification of FoF1-ATPase as a requirement for Bax to induce cell death in both yeast and mammalian cells.

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FOREWORD

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(5) INTRODUCTION

Bcl-2 is a proto-oncogene whose primary effect is not in stimulating cell proliferation; instead Bcl-2 functions as a cell death blocker. Bcl-2 promotes tumorogenesis by preventing cancer cells to die. Thus, understanding the precise mechanism of how Bcl-2 functions to prevent cell death is crucial to the understanding and treatment of human cancers, including breast cancer.

Bcl-2 belongs to a family of proteins that share extensive sequence homology. Although all appear to play some roles in regulating programmed cell death (PCD), some members of these family have opposing effects on cell death; Bcl-2 and Bcl-XL, for example, promote cell survival whereas Bax and Bak promote or induce cell death. The functions of Bcl-2 family proteins are also conserved during evolution. A Bcl-2 homologue, Ced-9 was identified in the nematode, *C. elegans*; overexpression of Bcl-2 can compensate for the defect of *ced-9* mutant worm (Hegnarter and Horvitz, 1994; Vaux et al., 1992). Moreover, ectopic expression of Bcl-2 can also rescue *sod* mutant yeast from oxidative injury (Kane et al., 1993), suggesting that Bcl-2 plays an evolutionarily conserved role in regulating life and death of the cell. In addition to acting in multicellular organisms to regulate PCD, some members of Bcl-2 family protein also retain at least some of their death regulatory roles in the unicellular yeast. Expression of either Bax or Bak imposes a lethal effect upon two yeast species, the budding yeast *S. cerevisiae* and the fission *yeast S. pombe* . On the other hand, Bcl-2 or Bcl-XL, mammalian cell death blockers also function in yeast to antagonize the cell death induced by Bax or Bak (Sato et al., 1994; Hanada et al., 1995; Zha et al., 1996; Greenhalf et al., 1996; Jürgensmeier et al., 1997; Ink et al., 1997; Tao et al., 1997).

The findings that Bcl-2 family proteins function as cell death inducers and blockers, respectively, in yeast prompted us to explore the yeast system for the study of PCD. We have developed a functional screen to search for mammalian genes that can inhibit Bax-induced cell death in yeast. From this screen, we have identified two human genes, named BI-1 and BI-2 (for Bax-Inhibitor 1 and 2). Cloning of these two genes and the initial characterization has been described in our previous annual report. In the past year we have extended these studies, attempting to determine the function and the underlying mechanisms for BI-1 and BI-2 functions. In addition, in collobarating with another postdoctoral associate in the lab, Dr. Shigemi Matsuyama, we also isolated Bax-resistant mutant yeast aimed at addressing the question of how Bax functions in yeast. We have cloned one yeast protein, *ATP4*, a subunit of the proton pump, these studies were further extended into mammalian cells, demonstrating that proton-pump is at least partially required for Bax to kill both yeast and mammalian cells. Our progress over the past year is summarized in this report.

(6) BODY

Progress on BI-1.

1. Overexpression of BI-1 inhibits drug-induced apoptosis in FL5.12 cells.

In addition to protecting cells against growth-factor deprivation, Bcl-XL also renders cells more resistant to some chemotherapeutic drugs (Minn et al., 1995). To determine if BI-1 has a similar function, the BI-1-stable FL5.12 clone (C12) that displayed strong protection against IL-3 deprivation was used for the drug-sensitivity test (Xu and Reed, 1998; also see 1997 Annual Report). As shown in Figure 1, overexpression of BI-1 rendered FL5.12 cells more resistant to etoposide, a topoisomerase II inhibitor and staurosporine, a kinase inhibitor. The drug concentrations used here were prior determined by dose titration to be effective at killing FL5.12 parental cells. Apoptosis assay was performed as described in Minn et al., 1995. Thus, all our data suggest that BI-1 inhibits apoptosis induced by various stimuli.

2. BI-1 does not affect Fas-induced apoptosis.

To address the question of if BI-1 functions only in the Bcl-2/Bax cell death pathway, we determined if BI-1 had any effect on Fas induced apoptosis by employing a transient transfection assay using 293 cells. It has been shown before that transient transfection of the Fas ligand-encoding plasmid into 293 cells induces apoptosis. BI-1 did not inhibit Fas-induced apoptosis, similar to Bcl-2 (Figure 2). As a positive control, an apoptosis inhibitor XIAP strongly inhibited Fas-induced apoptosis in the same experiment. Thus, the function of BI-1 is more restricted to the Bcl-2/Bax cell death pathway.

3. Antisense inhibition of BI-1 induces apoptosis.

The above functional data indicate that overexpression of BI-1 can inhibit apoptosis caused by a wide range of stimuli, suggesting that the BI-1 functions to promote cell survival. If this is the case, then cells may not be able to survive when BI-1 function is compromised. We tested this hypothesis by employing an antisense approach. It has been well documented that antisense DNA can reduce the expression of the corresponding gene. The complete BI-1 cDNA was cloned into pcIneo vector (containing a 5' intron that renders transcripts more stable) in reversed orientation. The resulting plasmid was transiently transfected into 293 cells, together with a GFP-marker plasmid to visualize transfected cells. To demonstrate that antisense BI-1 construct specifically inhibited the production of BI-1 protein; a Flag-tagged BI-1 plasmid was co-transfected with BI-1-AS into 293 cells. As shown in Figure 3B, the expression of BI-1-Flag decreased significantly in BI-1-AS transfected cells. The effect of antisense BI-1 was also demonstrated by its ability to inhibit translation of BI-1 *in vitro* (data not shown). In a dose dependent manner, anti-sense BI-1 induced apoptosis as indicated by the appearance of fragmented or condensed nuclei. Among transfected cells, about 20% were undergoing apoptosis, significantly higher than the control population with only less than 3% apoptotic cells (Figure 3A). Despite that the apparent effect of the antisense BI-1 was not as profound as some apoptosis stimuli, these data nonetheless suggest that BI-1 plays an important role in cell survival, since antisense DNA can only inhibit *de novo* synthesis of the BI-1 protein without having nay effect on the pre-existing BI-1 protein. Thus, we demonstrate from both sides that BI-1 functions to promote cell survival or to inhibit apoptosis.

4. The interaction between BI-1 and Bcl-2 is mediated through the BH4 domain of Bcl-2.

To map the region of Bcl-2 which interacts with BI-1, we tested several Bcl-2 mutants for the ability to co-immunoprecipitate with BI-1. This was done essentially as described above; except plasmids containing mutant Bcl-2 were used in transfection to substitute for the wild-type Bcl-2. Deletion of either the transmembrane domain (TM), Helix 2 (BH3) or the Helices 5 and 6 (part of BH1 and BH2) did not affect the binding capacity to BI-1 (data not shown). In contrast, deletion of the BH4 domain of Bcl-2 abolished the interaction between Bcl-2 and BI-1 (Figure 4), suggesting that the BH4 domain of Bcl-2 mediates the interaction between Bcl-2 and BI-1. This provides an interpretation for the ability of BI-1 to bind to Bcl-2 or Bcl-XL (both containing the BH4 domain), but not to Bax or Bak (both lacking the BH4 domain).

5. BI-1 antibody production

We have attempted to raise anti-BI-1 polyclonal antibodies in rabbits. We initially used an N-terminal peptide of BI-1, conjugated to KLH, and injected into rabbit. No antibody was produced that reacts with BI-1. We have also tried to use C-terminal BI-1 fused to the glutathione-S-transferase (GST), the GST-BI-1 fusion protein was produced *in E. coli*, purified, and injected into rabbit, but no immune response was mounted either. A likely reason for this is that BI-1 protein is overall extremely hydrophobic and thus not very antigenic. We will try to express BI-1 in insect cells using baculovirus expression system and used baculovirus-produced BI-1 protein as an antigen to immunize rabbits.

Progress on BI-2.

1. BI-2 inhibits Bax induced cell death in yeast.

BI-2 was isolated from a human HepG2 cDNA library by virtue of the ability to inhibit Bax-induced cell death in yeast (Details on HepG2 library screen are described in Xu and Reed, 1998). Inhibition of Bax-induced yeast cell death by BI-2 is shown in Figure 5A.

2. The C-terminus of BI-2 is required whereas the "Ring finger" is dispensable for Bax inhibitor activity of BI-2.

The BI-2 cDNA contains a predicted open reading frame of 1,350 bp, and encodes a protein of 450 amino acids. No gene identical to *BI-2* has been described before although cDNA fragments encompassing different regions of *BI-2* are found in the EST databases. Thus BI-2 is a novel gene. BLAST search revealed that BI-2 has a "Ring finger" motif in the N-terminal portion of the protein. Three hydrophobic stretches are also identified in BI-2. Furthermore, a region of BI-2 is weakly homologous to the "death effector domain" (DED).

BI-2 mutants deleting the "Ring finger" motif (BI-2ΔR) and missing the C-terminus (from amino acids 358-450; BI-2ΔC) were generated and cloned into the yeast expression vector p424GPD (ATCC, Rockland, MD). In yeast, BI-2ΔR can still protect against Bax-induced death, whereas BI-2ΔC has lost the Bax-inhibitor activity (Figure 5B).

3. BI-2 partially inhibits Bax induced apoptosis in 293 cells.

Having cloned BI-2 as a Bax inhibitor in yeast, the key question then was if BI-2 also functions similarly in mammalian cells. To address this question, we co-transfected BI-2 and Bax-encoding plasmid in the human embryonic kidney cells line, 293. As shown in Figure 6, in 293 cells, full-length BI-2 partially inhibited Bax-induced apoptosis in transient transfection assays (~ 50% reduction in percent of apoptosis). Thus, BI-2 functions to inhibit Bax induced cell death in both yeast and mammalian cells.

4. BI-2 mRNA is expressed in various human tissues at the mRNA level.

To examine the expression pattern of BI-2 in human tissues, Northern blot analysis was performed using poly(A)$^+$ RNA from eight different human tissues. As shown in Figure 7, BI-2 is expressed in all the human tissues examined, including heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas. Interestingly, two different size transcripts were detected for BI-1, with major mRNA of 3.0 kb and 1.5 kb. It remains to be determined if these different size transcripts arise from alternative splicing mechanisms and if they encode different proteins.

5. BI-2 is localized to intracellular membranes.

Bcl-2 and Bax are mostly in intracellular membranes, with majority of Bax in the mitochondria, whereas Bcl-2 is in the endoplasmic reticulum, nuclear envelope and mitochondria. BI-1, an inhibitor of Bax, identified through the same library screen as BI-2 was also found to be in intracellualr membranes (Xu and Reed, 1998). To preliminarily explore the intracellular location of BI-2, BI-2 was expressed in Cos-7 cells (a monkey kidney cell line) as GFP fusion, using the expression plasmid pEGFP-C2 which encodes a double mutant of GFP with brighter fluorescent properties than the wild-type GFP. Fluorescence microscopy demonstrated that BI-2 is almost exclusively associated with intracellular membranes in a pattern typical of the endoplasmic reticulum (ER) and its continuity with the nuclear envelope, although a small portion of BI-2 may be in Mitochodria.

6. BI-2 antibody production.

Using BI-2 N-terminus (amino acids 1-139) as an antigen (fused to GST), we have raised polyclonal antibodies against BI-2. This BI-2 antiserum recognizes *in vitro* translated full-length BI-2 and BI-2ΔR. It also recognizes cells overexpress BI-2ΔR.
We noticed that full-length BI-2 protein is only expressed at very low levels even when BI-2-encoding plasmid was transiently transfected into 293 cells. Interestingly, BI-2ΔR expresses very well, suggesting that the "Ring finger" region is involved in preventing BI-2 to be expressed at high levels. One possible explanation is that BI-2 is highly unstable and the "Ring finger" region of BI-2 mediates interaction with certain proteolysis machinery.

7. BI-2 interacts with Bcl-2 but not Bax.

To explore the mechanism of BI-2 action, we determined if BI-2 and Bcl-2 family proteins are in the same complex. We address this question by performing co-immunoprecipitation experiments. For this purpose, we co-transfected Bcl-2 with Myc-tagged BI-2ΔR (Myc-BI-2ΔR) since the full length BI-2 does not express well. (It should be noted that 293 cells have barely detectable endogenous Bcl-2, we therefore boosted up the level of Bcl-2 by transfecting in a Bcl-2 plasmid). 2 days after transfection, cells were lysed in lysis buffer containing 0.4 % NP-40 and immunoprecipitation was performed by diluting the extract to 0.2 % NP-40. Either mouse immunoglobulin (mIgG, as a negative control) or the anti-Myc monoclonal antibody 9E10 was used to pull down the Myc-BI-2ΔR immune complex. Western blot was then performed and probed with anti-Bcl-2 antiserum. As shown in Figure 8, Myc-tagged BI-2ΔR co-immunoprecipitated with Bcl-2, suggesting that these two proteins are indeed in the same complex. BI-2ΔR was also found to interact with Bcl-XL, a close relative of Bcl-2.

We performed similar experiments to determine if BI-1 interacts with Bax. In this case, 293 cells were co-transfected by Myc-tagged BI-2ΔR with Bax, lysed and immunoprecipitation was carried out as described above. We found that BI-2 failed to co-immunoprecipitate with Bax. A similar observation was made for BI-1; in other words, both BI-1 and BI-2 interact with Bcl-2 and Bcl-XL, but not Bax. There are several possible interpretations. It is possible that the interaction between BI-2 and Bax is fairly weak and it may be technically difficult to detect this interaction. Alternatively, BI-2 may indeed do not interact with Bax, and the observation that BI-2 can functionally inhibit Bax activity is mediated through a third protein.

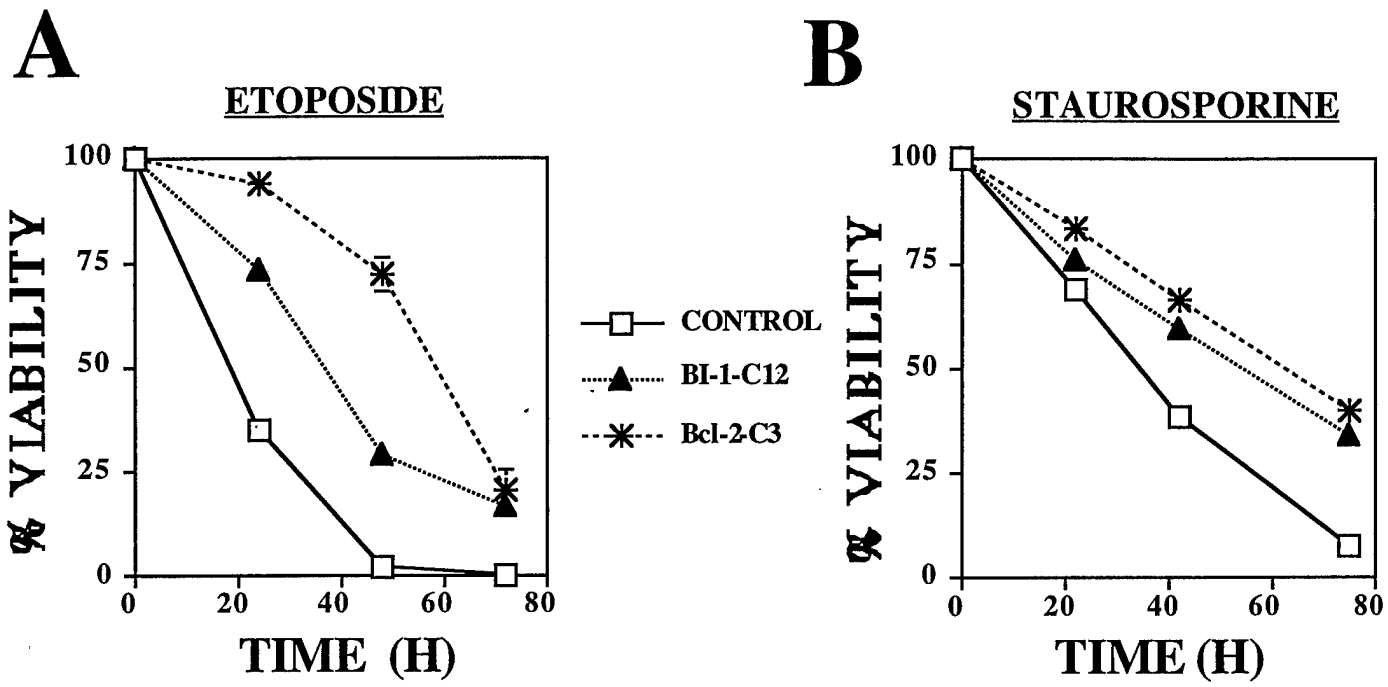

Figure 1. BI-1 protects against apoptosis induced by etoposide or staurosporine in FL5.12 lymphocytes.

FL5.12 stable clones were cultured in normal growth medium with either 5 μg/ml etoposide (A) or 0.5 μM staurosporine. The percentage of viable cells was determined at various times thereafter by trypan blue dye exclusion assay. Some SD bars are obscured by symbols.

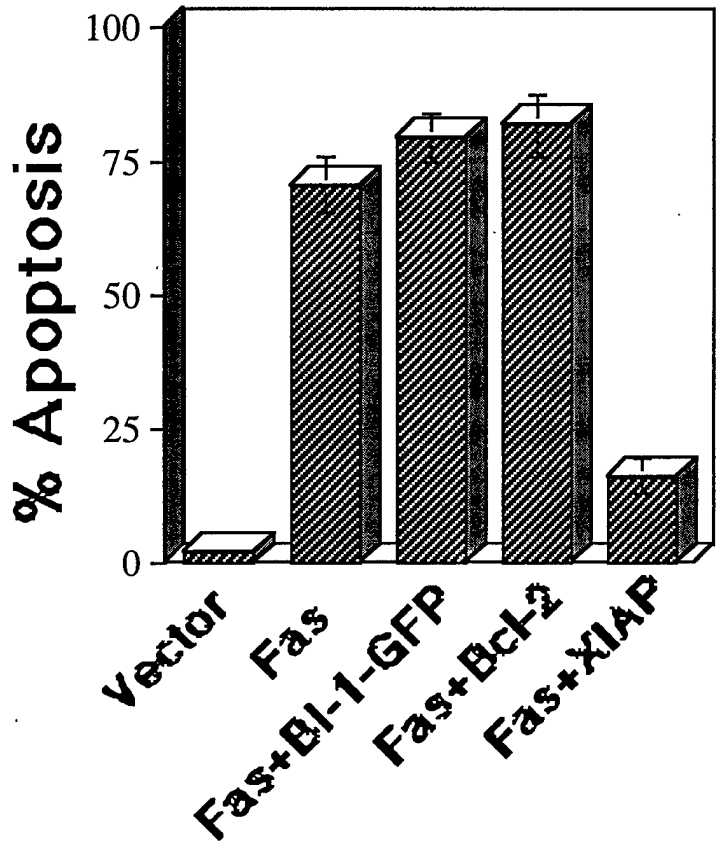

Figure 2. BI-1 has does not affect Fas-induced apoptosis

293 cells were transfected with either vector control or co-transfected with 0.5 μg of Fas-encoding plasmid and 8 μg of indicated plasmids. GFP-encoding plasmid (0.5 μg) was included in all transfections. 20-h after transfection, both floating and adherent cells (after trypsinization) were pooled, fixed, and stained with DAPI (Zha et al., 1996). The percentage of GFP-positive cells with fragmented nuclei (apoptotic) was determined.

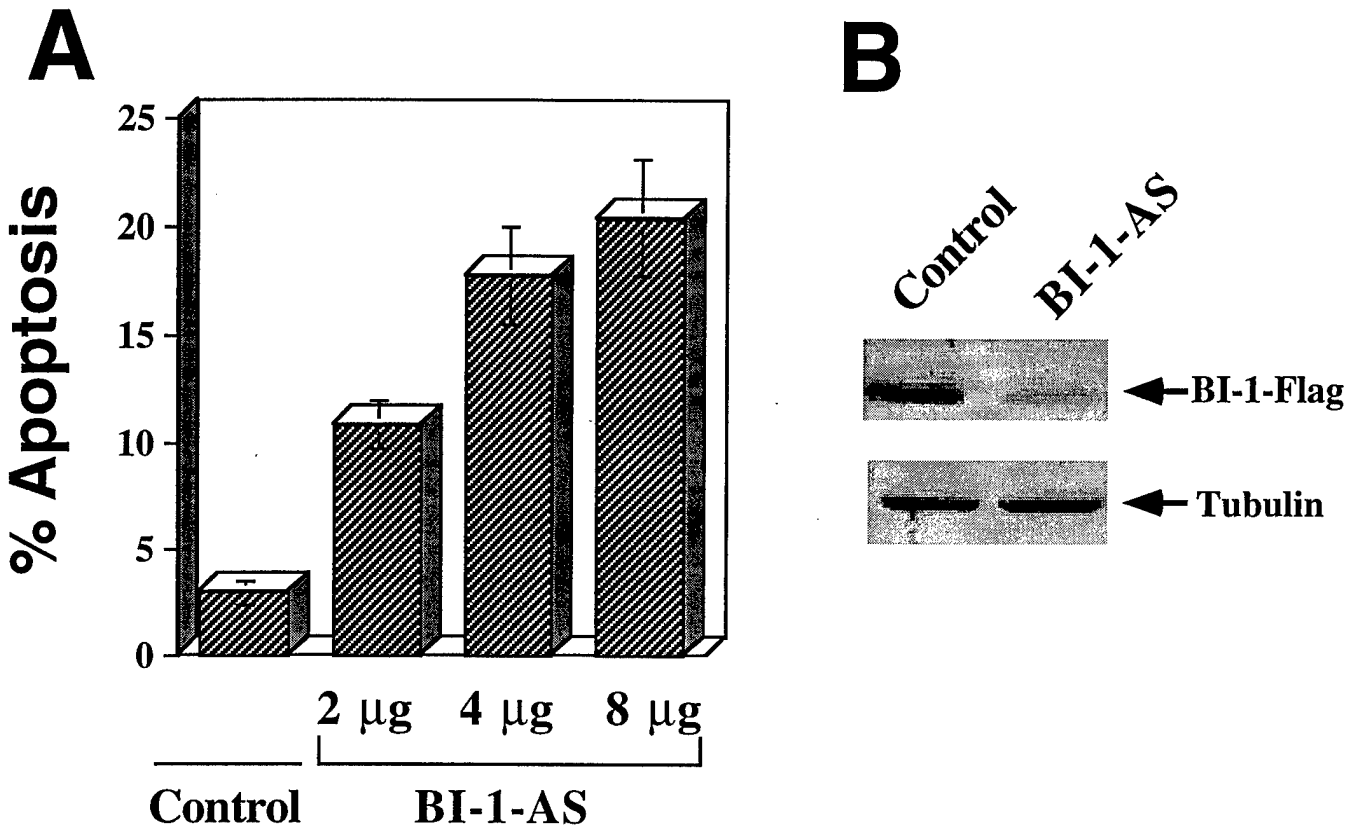

Figure 3. Antisense inhibition of BI-1 induces apoptosis.

(A) 293 cells were co-transfected with indicated plasmids plus 1 μg GFP-encoding plasmid. The total amount of plasmid used for each transfection was fixed at 9 μg. 30-h after transfection, both the floating and adherent cells were pooled, fixed and stained with DAPI. % apoptosis was determined as the ratio of cells with fragmented or condensed nuclei among transfected cells. Data shown represent three independent experiments (mean ± SD, n=3).
(B) Antisense BI-1 specifically inhibit expression of the BI-1 protein. 293 cells were co-transfected with 1 mg BI-1-Flag-encoding plasmid in combination with either 8 μg vector or antisense BI-1 plasmid (BI-1-AS). 30-h after transfection, protein extracts were prepared and subjected to SDS-PAGE and immunoblot analysis using the anti-Flag M2 antibody and an anti-tubulin antibody (served as a loading control).

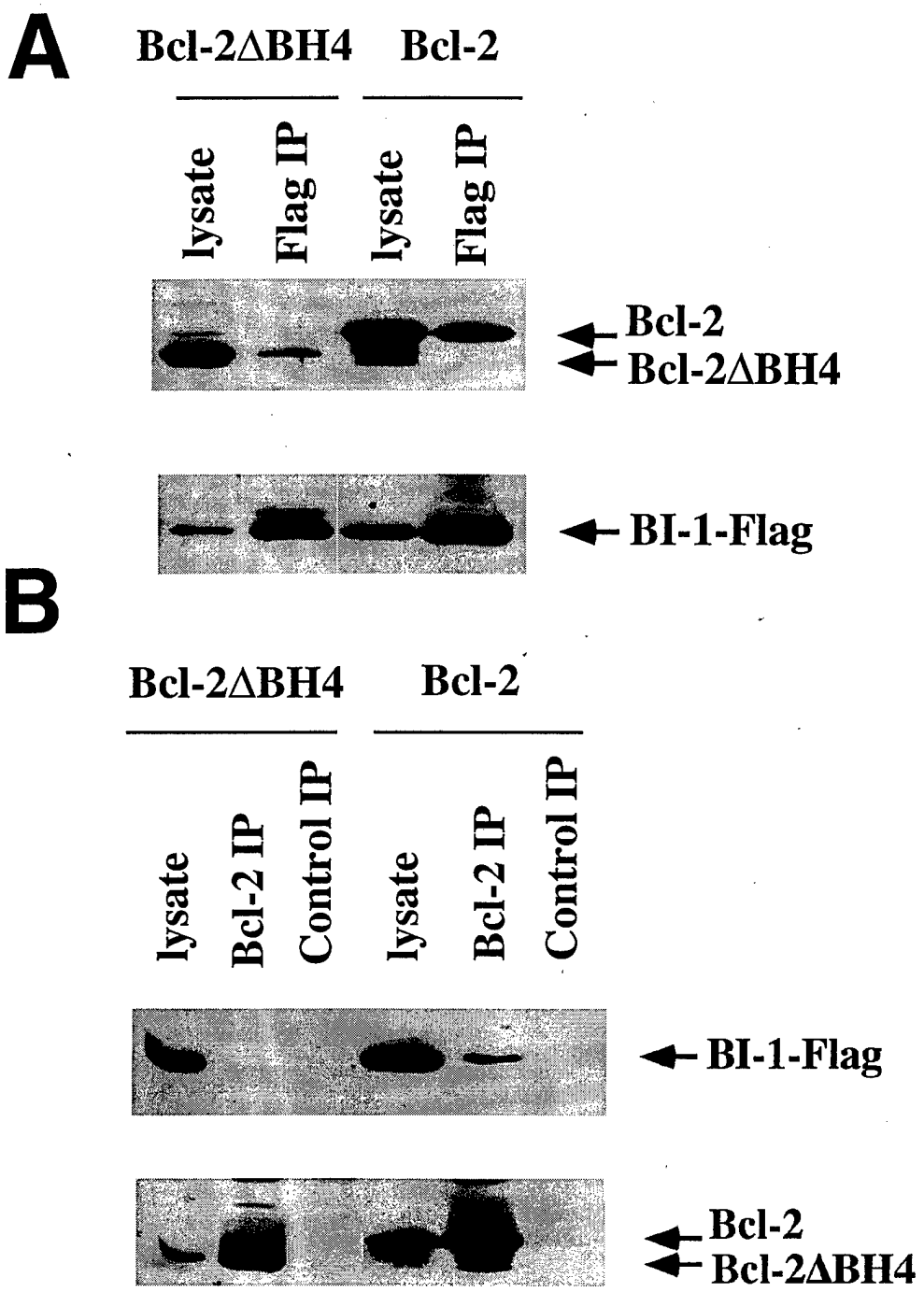

Figure 4. The BH4 domain of Bcl-2 mediates the interation between Bcl-2 and BI-1.

293 cells were transiently transfected with either Bcl-2- or Bcl-2ΔBH4-encoding plasmids together with either vector control plasmid or BI-1-Flag-encoding plasmid. 2 days after transfection, cells were lysed in 1% NP-40 buffer and immunoprecipitations were performed using either anti-Bcl-2 antiserum or preimmune rabbit serum. After extensive washing, immune complexes were heated at 42°C for 15 min. in SDS sample buffer and subjected to SDS-PAGE/immunoblot analysis using an antibody specific for FLAG (top panel) or Bcl-2 (bottom panel). "Lysates" from cells transfected with Bcl-2- or Bcl-2ΔBH4-encoding plasmids represented 1/20 input used for immunoprecipitation.

A

B

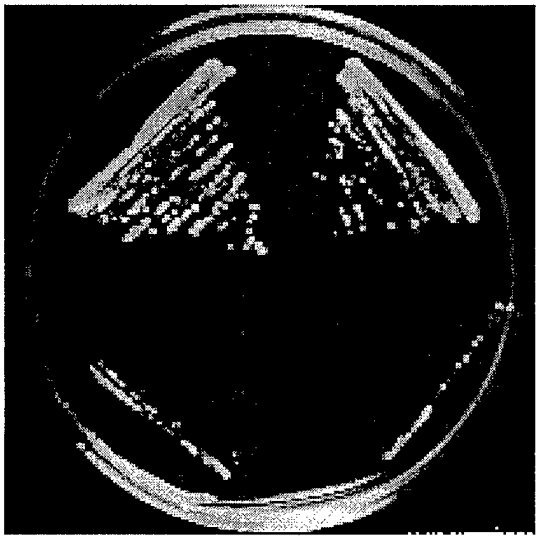

Figure 5. The effect of BI-2 and BI-2 mutants on Bax-induced cell death in yeast.

(A) Either control vector or BI-1-encoding expression plasmid (isolated from the HepG2 library) was transformed into cells of yeast strain QX95001 (harboring YEp51-bax). Transformants were streaked on galactose-containing synthetic medium lacking uracil and leucine. Photograph was taken after a 4-day incubation at 30°C.
(B) Yeast expression plasmids containing either wild-type BI-2 or mutant forms of BI-2 (BI-2ΔR and BI-2ΔC), or empty vector were transformed into cells of yeast strain QX95001 and analyze as described in (A).

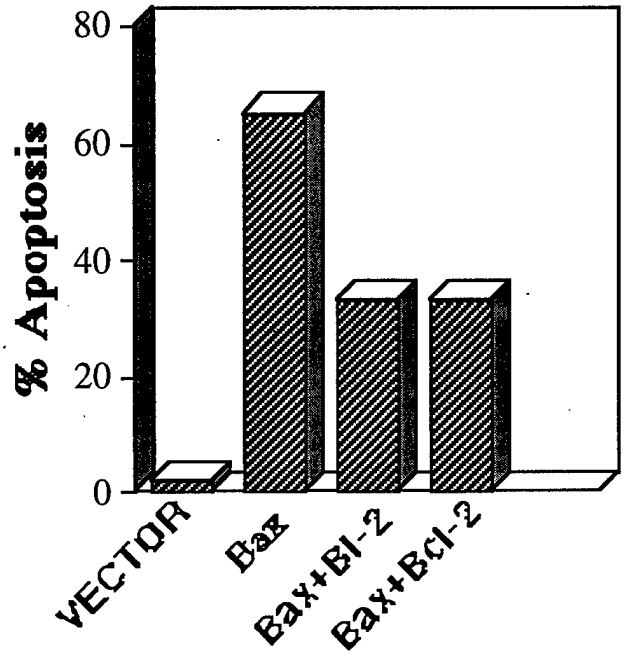

Figure 6. BI-2 protects against Bax-induced apoptosis in 293 cells.

293 cells were transiently transfected with 9 μg of vector control, or contransfected with 3 μg of Bax plasmid and 6 μg of either vector control, or BI-2, or Bcl-2 plasmid. 1 μg pEGFP-N2 vector was also included to visualize transfected cells. 24-h after transfection, both the floating and adherent cells were pooled, fixed and stained with DAPI. The percentages of apoptotic cells among transfected population were calculated.

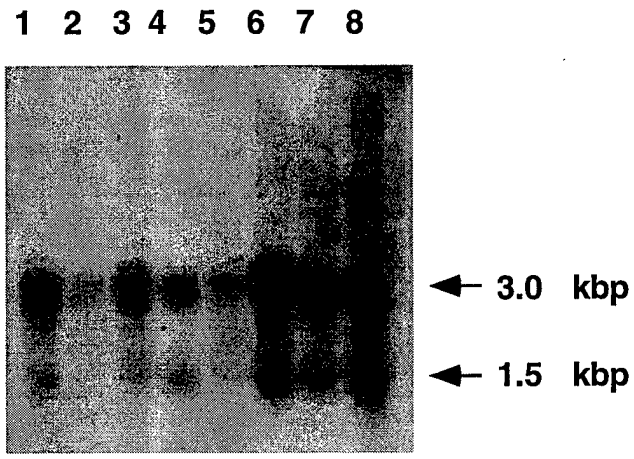

Figure 7. Tissue distribution of BI-2 mRNA.

Human poly (A)+ RNA derived from various tissues was analyzed by Northern blotting using a 0.7-kb fragment of BI-1 cDNA as a probe. Lanes 1-8 contain, in order, mRNA from human heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas.

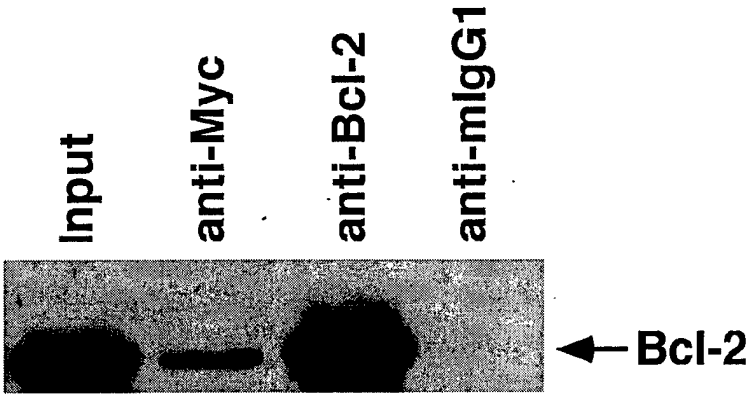

Figure 8. BI-2 interacts with Bcl-2.

293 cells were transiently transfected with plasmids encoding Bcl-2 and Myc-BI-2ΔR. 2 days after transfection, cells were lysed in 0.5 % NP-40 containing HKME buffer and immunoprecipitations were performed (after diluting NP-40 concentration to 0.2%) using the indicated antibodies. mIgG1 was included as a negative control. Immune complexes and input (representing ~ 1/25 of the lysates used for immunoprecipitation) were subjected to SDS-PAGE and immunoblot analysis using antisera for Bcl-2 and BI-2.

Genetic analysis of Bax-induced cell death in yeast.

In collaboration with another postdoctoral fellow in our laboratory, Dr. Shigemi Matsuyama, we have undertook a yeast genetic approach as described in the original proposal (Aim#4) aimed at uncovering yeast genes that are required for Bax-mediated lethality in *S. cerevisiae*. Through this analysis, we found that ATP4, a component of the FoF1 ATPase, is required for Bax killing of both yeast and mammalian cells. Our findings imply that an intact FoF1 ATPase in the inner Mitochondria membrane is necessary for optimal function of Bax in both yeast and mammalian cells.

1. **ATP4 is required for Bax-induced lethality in yeast.**

By mutagenizing yeast, we have isolated a number of yeast mutants that rescue yeast from Bax-induced cell death. Further genetic analysis of these mutants has identified one single-genic recessive mutation. We focused on this mutation and cloned the corresponding wild-type yeast gene by complementation (Matsuyama et al., 1998). Nucleotide sequence analysis revealed that this is the yeast *ATP4* gene, which encodes the subunit 4 of the yeast FoF1-ATPase, a proton-pump located in the inner membrane of mitochondria (Weber and Senior, 1997).

Since a mutation in *ATP4* renders yeast resistant to Bax-induced cell death, we further addressed the question if *ATP4* is required for Bax-induced killing of yeast by utilizing the yeast strain PVY10 with disrupted *ATP4* gene (Velours et al., 1989). To this end, PVY10 yeast cells were transformed by *bax*-containing plasmid and empty vector control. Yeast cells were grown in galactose-containing medium to induce Bax expression and cell viability was determined by a trypan blue dye exclusion assay (Matsuyama et al., 1998). As shown in Figure 9A, *ATP4* knockout yeast was indeed resistant to Bax killing. Immunoblot analysis demonstrated that the *ATP4* mutation did not affect production of Bax protein (data not shown). Thus, the *ATP4* gene is required for Bax-mediated killing of yeast.

2. **Oligomycin, a proton-pump inhibitor, inhibits Bax-mediated yeast cell death.**

Oligomycin binds to the Fo portion of the yeast and mammalian FoF1 ATPase and thus effectively shuts off the proton pump (Tzagoloff, 1970). We reason that if the proton pump is required for Bax-mediated lethality in yeast, then turning off the proton pump by oligomycin should render wild-type yeast resistant to Bax. This hypothesis was tested by treating Bax-containing yeast cells with oligomycin. As shown in Figure 9B, oligomycin partially inhibited Bax-induced killing of yeast. Under these conditions, oligomycin did not inhibit the growth of yeast due to their ability to produce sufficient ATP via anaerobic fermentation (not shown). Therefore, similar to disruption of the *ATP4* gene, a pharmacological inhibitor of the FoF1 ATPase proton pump also inhibits Bax-induced cell death in yeast.

3. **Oligomycin also inhibits Bax-induced apoptosis in mammalian cells.**

We further determined if the proton pump is also required for optimal function of Bax in mammalian cells. No mammalian cells exist that harbor mutations within subunits of the mitochondrial FoF1 ATPase. Thus we employed pharmacological intervention using oligomycin. Differing from yeast, in mammalian cells oligomycin is toxic and leads to either

apoptosis or necrosis, depending on cell type and circumstances evaluated (Castedo et al., 1996). Cell death caused by oligomycin, however, can be delayed by increasing the concentration of glucose in the culture medium, which helps to maintain ATP levels through glycolysis (Eguchi et al., 1997; Leist et al., 1997). We determined the effect of oligomycin in human 293T kidney epithelial cells grown in high glucose medium, using a transient Bax transfection assay to induce apoptosis (Zha et al., 1996). For all experiments, oligomycin was added 4 hours after transfection, apoptosis was measured after an additional 8 hours of culturing. Thus, the experiments were performed within the first 12 hour after Bax transfection, before oligomycin caused cell death. As shown in Figure 10, oligomycin reduced the percentage of apoptotic cells in a dose dependent manner. In contrast, antimycin A, an inhibitor of the respiratory complex III, did not impair Bax induced apoptosis under these conditions, suggesting that proton pump is required for Bax-mediated lethality, whereas respiration is not. We also found that oligomycin inhibited Bax-induced caspase activation under these conditions (Figure 10B). Thus, based on these results, we conclude that the FoF1 ATPase is either required for the optimal function of Bax in 293 T cells or enhances the ability of Bax to induce apoptosis in these human cells.

4. **Oligomycin inhibits p53-induced apoptosis.**

We also examined the effect of oligomycin on Bax induced apoptosis in a different cellular context. In baby rat kidney (BRK) cells, the induction of apoptosis by p53 has been shown to be Bax-dependent (Han et al., 1996; Sabbatini et al., 1997). We therefore employed BRK cells that express a temperature-sensitive mutant of p53, and examined the impact of oligomycin on p53-induced apoptosis in these cells. As in the prior experiments, these cells were grown in high glucose medium to maintain ATP levels via glycolysis. As shown in Figure 11A, oligomycin reduced the percentage of apoptotic cells when BRK cells were cultured at 32 °C to activate p53. In contrast, antimycin A had no apparent effect on p53-induced apoptosis under these conditions. Western blot analysis showed that neither oligomycin nor antimycin A impaired p53-induced expression of Bax when cells were grown at the permissive temperature of 32 °C (Figure 11B). Taken together, these data provide further evidence that Bax-dependent apoptosis requires the mitochondrial FoF1 ATPase proton pump.

In the past year, we have accomplished tasks 18-21 (Aim#4) proposed in the original proposal, although further studies are warranted to determine the underlying mechanisms by which FoF1 ATPase impinges upon cell death regulation. We have either accomplished or partially accomplished tasks 22-28 in the revised STATEMENT OF WORK.

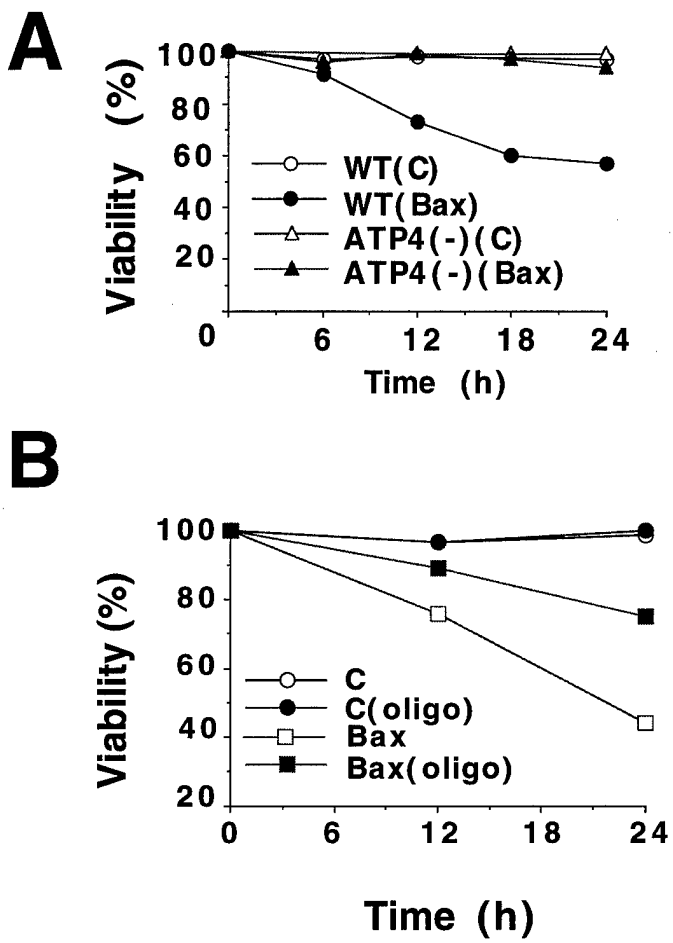

Figure 9. Yeast with inactive FoF1 ATPase are resistant to Bax-induced cell death.

(A) Yeast with disrupted ATP4 gene are resistant to Bax-induced cell death.
Wild-type (WT) yeast, *ATP4* knockout yeast (ATP4-) were transformed with pGilda (C) or pGilda-Bax (Bax). The cells were grown in glucose-based medium and then transferred to galactose-based medium to induce Bax expression from the *GAL1* promoter in pGilda plasmids. The percentage of trypan blue dye excluding cells was determined at various times after transferring to galactose-containing medium.
(B) F0F1-ATPase proton-pump inhibitor, oligomycin, attenuates Bax-induced Cell death in yeast. Wild-type yeast containing either pGilda (C) or pGilda-Bax (Bax) were initially cultured in glucose-containing medium, and then transferred to galactose-containing medium with (closed symbols) or without (open symbols) 10 μM oligomycin, and the percentage of trypan blue dye excluding cells was determined at various times thereafter.

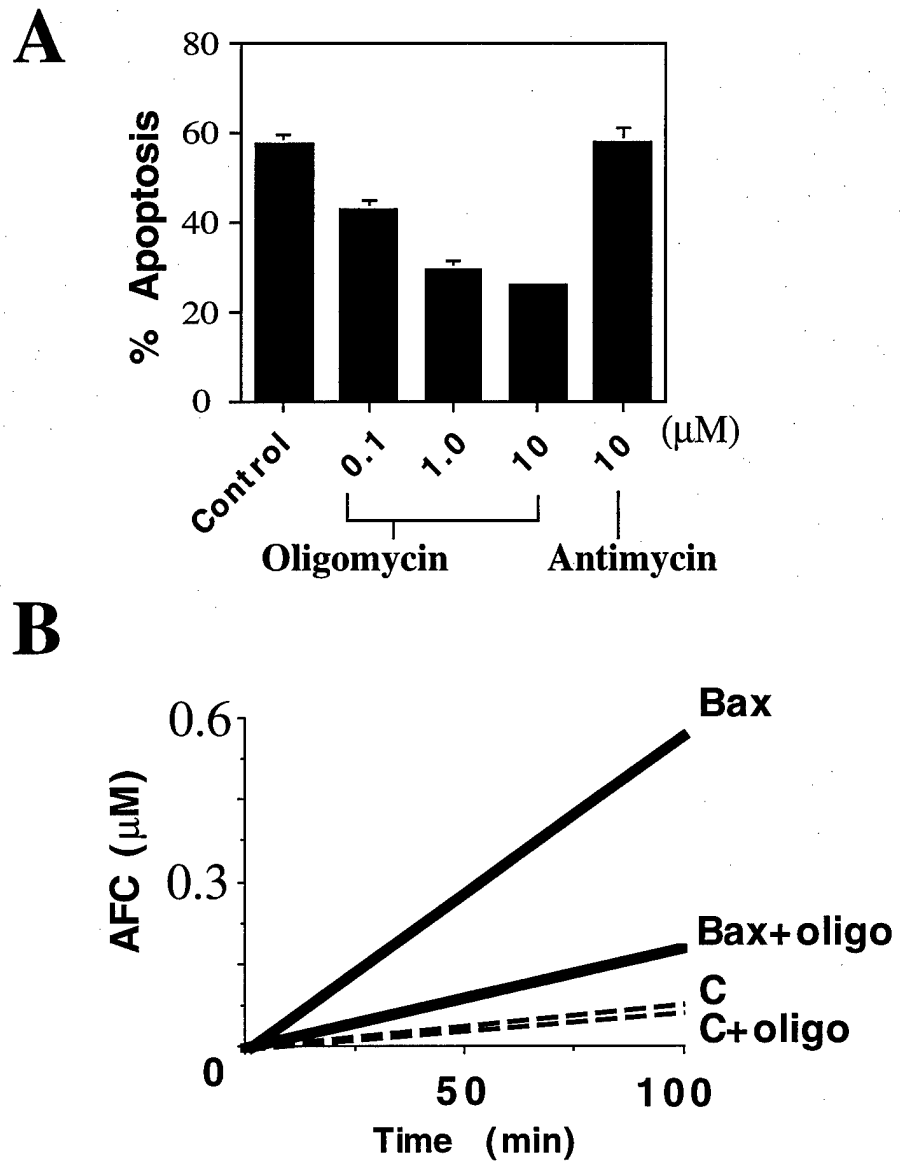

Figure 10. The FoF1 ATPase inhibitor oligomycin suppresses Bax-induced apoptosis and caspase activation.

(A) 293T cells were cultured in DMEM-high glucose medium to maintain ATP levels by glycolysis. 4 hours after transfection with 9 μg of pcDNA-Bax (Bax) or control pcDNA (C) plasmid with 1 μg of pEGFP, fresh medium containing or lacking 0, 0.1, 1, or 10 μM oligomycin or 10 μM antimycin A was used to replace the old culture medium. After an additional 8 hour of culture, the cells were collected and the percentage of GFP positive cells with apoptotic morphology was determined by DAPI-staining.

(B) Lysates derived from cells that had been cultured with or without 10 μM oligomycin were prepared and normalized for total protein content, and caspase activity was measured based on hydrolysis of DEVD-AFC (Deveraux et al., 1997).

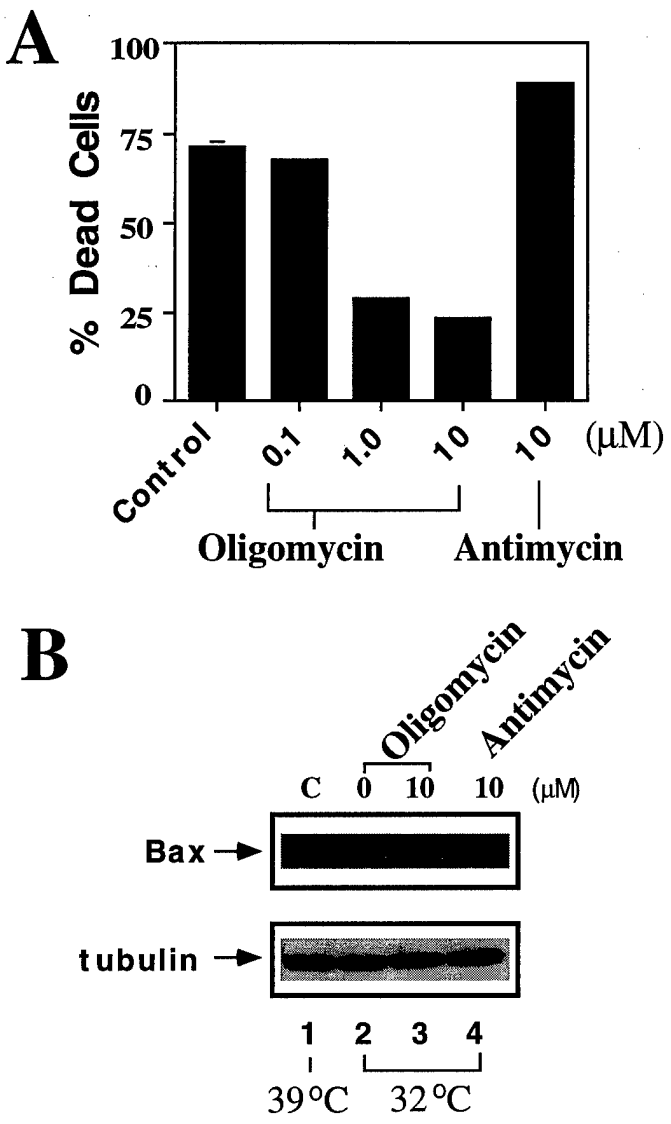

Figure 11. Oligomycin inhibits p53-induced apoptosis.

BRK cells that contain temperature-sensitive p53 (Han et al., 1996) were maintained at a nonpermissive temperature of 39 °C, then cultured at 32 °C to induce p53 in the presence or absence of 0.1-10 μM oligomycin or 10 μM antimycin A.

(A) the percentage of dead cells was determined by DAPI staining 12 hours after shifting to 32 °C.

(B) Lysates were prepared from BRK cells that had been cultured at 39 °C (lane1) as a control (C) or at 32 °C (lane 2-4) with or without 10 μM oligomycin or 10 μM antimycin A for 12 hours. 5 μg total protein was loaded on a protein gel and analyzed by immunoblotting using anti-Bax antiserum with ECL-based detection. The same blot was reprobed with anti-tubulin to demonstrate equal loading.

(7) CONCLUSIONS

Previously we have cloned BI-1 and BI-2 as Bax inhibitors. Over the past year, we have furthered our studies on BI-1 and found that BI-1 can inhibit apoptosis induced by a wide variety of stimuli. In addition to overexpression studies, we have performed antisense ablation studies and found that decreasing BI-1 protein level sensitizes 293 cells to undergo apoptosis. We have mapped the domain on Bcl-2 required for the interaction with BI-1, providing explanation for the previous observation that BI-1 interacts with Bcl-2 and Bcl-XL (both containing the BH4 domain), but not Bax and Bak (both lacking the BH4 domain). We have started characterization of BI-2 and found that BI-2 can at least partially inhibit Bax-induced apoptosis in human 293 cells. Similar to BI-1, BI-2 is localized to intracellualr membranes and interacts with Bcl-2 and Bcl-XL, but not Bax. Genetic studies of Bax-mediate lethality also progressed well and led to the identification of the FoF1-βATPase as a critical requirement of Bax to kill yeast. These studies resulted in two back-to-back publications in the journal Molecular Cell (a new companion journal to Cell). These papers are attached in Appendix.

Future directions involve the determination of the effect of BI-2 on apoptosis induced by a variety of other stimuli, similar to studies that were carried out for BI-1. The presence of a potential "DED" death effector domain raised the intriguing possibility that BI-2 may functionally bridge between Bcl-2 and "DED"-containing caspases. Our preliminary data suggest that BI-2 specifically interacts with some caspases but not others. We are currently pursuing these. We will also address the possibility of BI-1 and BI-2 as potential targets of anti-cancer drugs; initially we will compare the expression of BI-1 and BI-2 in normal and cancer cell lines and tissues by performing immunoblot analysis and immunohistochemistry studies.

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Bax Inhibitor-1, a Mammalian Apoptosis Suppressor Identified by Functional Screening in Yeast

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Summary

The mammalian proapoptotic protein Bax confers a lethal phenotype when expressed in yeast. By exploiting this phenotype, we have identified a novel human Bax inhibitor, BI-1. BI-1 is an evolutionarily conserved integral membrane protein containing multiple membrane-spanning segments and is predominantly localized to intracellular membranes, similar to Bcl-2 family proteins. Moreover, BI-1 can interact with Bcl-2 and Bcl-X_L but not Bax or Bak, as demonstrated by in vivo cross-linking and coimmunoprecipitation studies. When overexpressed in mammalian cells, BI-1 suppressed apoptosis induced by Bax, etoposide, staurosporine, and growth factor deprivation, but not by Fas (CD95). Conversely, BI-1 antisense induced apoptosis. BI-1 thus represents a new type of regulator of cell death pathways controlled by Bcl-2 and Bax.

Introduction

Bcl-2 family proteins are centrally involved in control of programmed cell death (PCD), with some inhibiting (Bcl-2 and Bcl-X_L) and others promoting (Bax and Bak) apoptosis (reviewed in Reed, 1994; Yang and Korsmeyer, 1996; Kroemer, 1997). The ability of Bcl-2 family proteins to regulate cell life and death is conserved across evolution. For example, the nematode *Caenorhabditis elegans* contains a Bcl-2 homolog, CED-9, that is essential for the viability of these animals, and expression of the human Bcl-2 protein in *C. elegans* can rescue CED-9-deficient worms (Vaux et al., 1992; Hengartner and Horvitz, 1994). The human Bcl-2 protein can also block apoptotic cell death in insect cells (Alnemri et al., 1992), and human Bcl-2 can protect some mutant yeast strains from death induced by oxidative injury (Kane et al., 1993).

The biochemical mechanism of action of Bcl-2 and its homologs is controversial (reviewed by Reed, 1997a). Recent determination of the three-dimensional structure of Bcl-X_L suggests similarity to the pore-forming domains of some bacterial toxins, particularly diphtheria toxin and the colicins (Muchmore et al., 1996). These toxins function by forming channels in membranes that transport either ions or proteins (Donovan et al., 1981; Cramer et al., 1995). Several members of the Bcl-2 family, including Bcl-2, Bcl-X_L, and Bax, are capable of forming ion-conducting channels in synthetic membranes in vitro (Antonsson et al., 1997; Minn et al., 1997; Schendel et al., 1997; Schlesinger et al., 1997). Thus, some Bcl-2 family proteins may have intrinsic activities as channel proteins, irrespective of other functions related to their ability to interact with several types of proteins in cells (Reed, 1997a).

The mammalian Bax protein confers a lethal phenotype when expressed in either the budding yeast *Saccharomyces cerevisiae* or the fission yeast *Schizosaccharomyces pombe* (Sato et al., 1994; Greenhalf et al., 1996; Zha et al., 1996; Ink et al., 1997; Jürgensmeier et al., 1997). The cell death induced by Bax or its close relative Bak is not attributable to nonspecific toxicity caused by overexpression of a heterologous protein, since antiapoptotic Bcl-2 proteins can rescue yeast from Bax/Bak-induced lethality. Moreover, mutants of Bcl-2 and Bcl-X_L that fail to protect in mammalian cells are inactive at suppressing Bax-induced cell death in yeast. In addition, certain mutations of Bax and Bak that abolish their proapoptotic function in mammalian cells also abrogate their lethal effects in yeast (Zha et al., 1996; Ink et al., 1997). Recently, Bax has been shown to induce release of cytochrome c from mitochondria in yeast (Manon et al., 1997), which further suggests commonalities in the mechanisms by which Bax induces death in yeast and mammalian cells, given that apoptosis is often associated with release of cytochrome c from mitochondria in mammalian cells (reviewed by Reed, 1997b). In addition, a requirement for the F_0F_1-ATPase proton pump of mitochondria has been demonstrated for optimal cell death-inducing function of Bax in both yeast and mammalian cells (Matsuyama et al., 1998 [this issue of *Molecular Cell*]).

By exploiting the lethal phenotype of Bax in yeast, we reasoned that it should be possible to identify mammalian proteins that suppress Bax function by screening cDNA expression libraries for clones that rescue yeast from Bax-mediated cell death.

Results

cDNA Cloning of Human BI-1

A yeast strain (QX95001) was constructed by transforming the Bax-expression plasmid YEp51-Bax, encoding the full-length mouse Bax protein under the control of the galactose-inducible yeast *GAL10* promoter (Zha et al., 1996), into strain BF264-15Dau (Lew et al., 1991). These yeast cells died upon transfer from glucose to galactose-containing medium, which induces the *GAL10* promoter in this plasmid and leads to accumulation of Bax protein in yeast (Zha et al., 1996). A human HepG2 cDNA expression library (Lew et al., 1991) was transformed into the QX95001 strain and screened for Bax-resistant transformants by plating on galactose-containing solid medium. From a screen of $\sim 10^6$ transformants, 17 displayed Bax resistance. Of these, 4 were determined to be dependent on the introduced cDNA-library plasmid by "con-commitant-loss" assay (Ausubel et al., 1991). The nucleotide sequences of 3 of these cDNA clones encoded the same protein, designated

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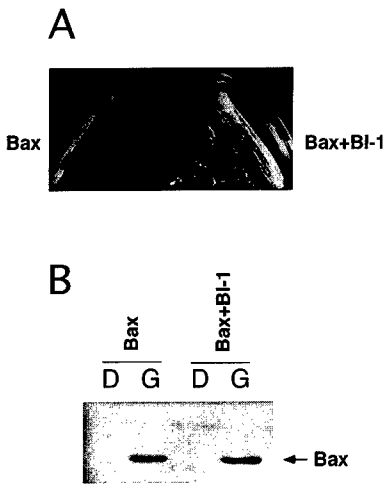

Figure 1. Bl-1 Suppresses Bax-Induced Yeast Cell Death

Either control vector or Bl-1-encoding expression plasmid (isolated from the HepG2 library) was transformed into cells of yeast strain QX95001 (harboring YEp51-Bax).

(A) Transformants were streaked on galactose-containing synthetic medium lacking uracil and leucine. Photograph was taken after a 4 day incubation at 30°C.

(B) Protein extracts were prepared from QX95001 transformants used in (A) that contained control or Bl-1-encoding plasmids. Cells were grown in glucose-containing medium (D = dextrose) and then transferred to galactose-containing medium (G) for 20 hr. Total protein extracts (20 μg/lane) were subjected to SDS–PAGE and immunoblot analysis using anti-mBax antiserum.

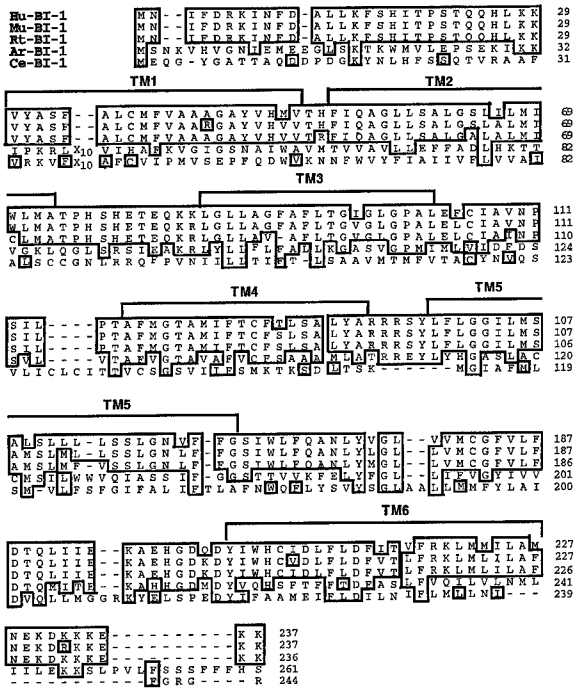

Figure 2. Bl-1 Is an Evolutionarily Conserved Protein with Several Predicted Membrane-Spanning Domains

The predicted amino acid sequences of human, rat, and mouse Bl-1-proteins, as well as the homologous *C. elegans* and *Arabidopsis* proteins are aligned, with identical residues in boxes. The predicted 6 TM domains of the human Bl-1 protein are indicated in brackets.

Bl-1, for Bax Inhibitor-1. Bl-1 did not interfere with production of the Bax protein in yeast, as determined by immunoblot analysis (Figure 1).

The Predicted Bl-1 Protein Contains Several Membrane-Spanning Segments

All three Bl-1 cDNAs obtained by the functional yeast screen contained an open reading frame (ORF) encoding a predicted protein of 237 amino acids. The predicted AUG start codon for this ORF was within a favorable context for translation initiation (Kozak, 1997) and was preceded by an in-frame stop codon. A search against the available nucleotide sequence databases using the BLAST program (Altschul et al., 1990) revealed that Bl-1 is essentially identical to TEGT ("testis enhanced gene transcript"), a cDNA previously cloned fortuitously during an attempt to identify testis-specific genes (Walter et al., 1995). The rat TEGT protein has also been described and shares 90% identity (95% similarity) with the human protein (Walter et al., 1994). Additional previously undescribed homologs of Bl-1 were also identified by BLAST searches in mouse, and possibly in the nematode *C. elegans* and the plant *Arabidopsis thaliana*. The mouse Bl-1 protein, as deduced from ESTs #AA015124, AA275830, AA467259, AA107704, and W59401 is 237 amino acids in length and shares 92% identity (95% similarity) with the human Bl-1 protein (Figure 2). The *C. elegans* ORF (EMBL #Q20241) encodes a protein of 241 amino acids that shares 21% overall amino acid identity (37% similarity) with human Bl-1. This percentage homology shared by the nematode and mammalian Bl-1 is similar to that reported for Ced-9 and Bcl-2, which share only 23% identity in their amino acid sequences. The putative *Arabidopsis* homolog (EMBL #Z97343) is 261 residues in length, sharing 29% amino acid sequence identity (45% similarity) with the human Bl-1 protein.

Based on Kyte-Doolittle hydropathy analysis (Kyte and Doolittle, 1982), the Bl-1 protein is predicted to contain 6 or 7 transmembrane (TM) domains. The C termini of the mammalian Bl-1 proteins are comprised of basic amino acids resembling some nuclear targeting sequences (Dingwall and Laskey, 1991), but otherwise the predicted proteins lack motifs that suggest a function. Of note, Bl-1 does not contain any of the conserved BH domains of Bcl-2 family proteins. By searching a transmembrane protein database (TMbase) using the TMpred program (Hofmann and Stoffel, 1993), the most favored membrane topology for mammalian Bl-1 is with 6 membrane-spanning domains and both the N and C termini oriented toward the cytosol. The putative transmembrane segments are predicted to assume mostly an α-helical conformation. Triton X-114 partitioning studies confirmed that Bl-1 is an integral membrane protein (data not presented). RNA blot analysis indicated that Bl-1 is widely expressed in vivo, including heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas (not shown).

Bl-1 Inhibits Bax-Induced Apoptosis in Mammalian Cells

Transient transfection of Bax-encoding expression plasmids induces apoptosis in the human embryonic kidney

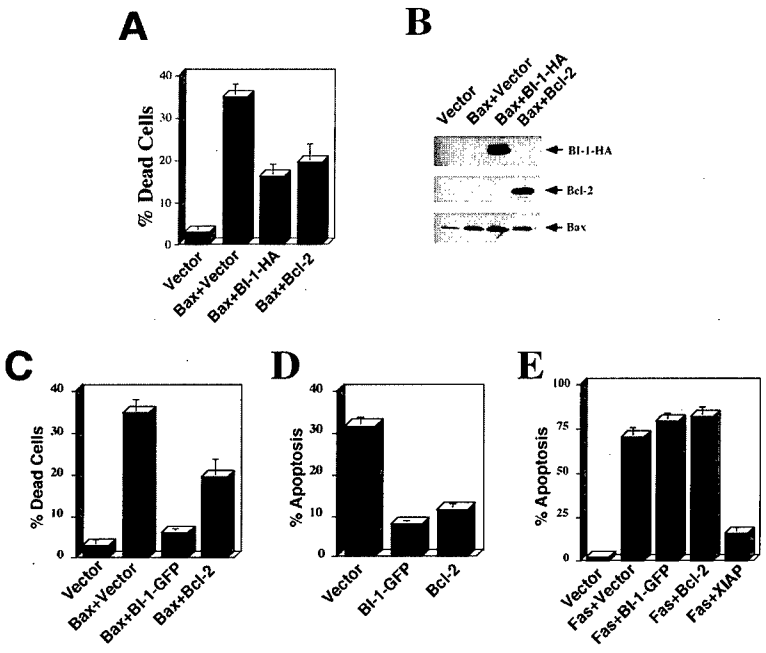

Figure 3. BI-1 Inhibits Cell Death Induced by Bax or Serum Withdrawal but Not Fas

(A) BI-1 protects against Bax-induced apoptosis in 293 cells. 293 cells were transfected with either 9 μg of vector control, or cotransfected with 3 μg of Bax plasmid and 6 μg of either control vector (pcDNA3) or plasmids encoding BI-1 (with a C-terminal HA tag) or Bcl-2. One day after transfection, floating cells and adherent cells (after trypsinization) were pooled. A portion of the pooled cells was subjected to vital dye trypan blue exclusion assay counting at least 300 cells (mean ± SD; n = 3).

(B) Extracts were prepared from another portion of the transiently transfected 293 cells described in (A) above and subjected to SDS-PAGE/immunoblot analysis. The blot was sequentially probed with anti-HA monoclonal antibody, anti-hBax antiserum, and anti-Bcl-2 antiserum, with stripping between each detection. The predicted band corresponding to the ≈30 KD BI-1-HA protein is indicated with an arrow.

(C) 293 cells were transiently transfected with either vector control (9 μg) or cotransfected with 3 μg of Bax plasmid and 6 μg of either BI-1-GFP or Bcl-2 plasmid. Trypan blue dye positive cells (%) were determined 1 day later (mean ± SD; n = 3). The expression of GFP-tagged BI-1 was verified by fluorescence microscopy and by immunoblotting using an anti-GFP monoclonal antibody (now shown).

(D) GM701 cells were cotransfected with a β-gal reporter plasmid (0.5 μg) and the indicated plasmids (4.5 μg each). 18 hr after transfection, cells were washed and cultured in DMEM containing 0.1% FBS for another 30 hr. Floating and adherent cells were fixed and stained with X-gal. The percentage of blue cells (transfected) with apoptotic morphology was determined (mean ± SD; n = 3).

(E) 293 cells were transfected with either vector control (8.5 μg) or cotransfected with 0.5 μg of Fas-encoding plasmid and 8 μg of either vector, or BI-1-GFP-, or Bcl-2- or XIAP-encoding plasmids. GFP-encoding plasmid (0.5 μg) was included in all transfections. 20 hr after transfection, both floating and adherent cells (after trypsinization) were pooled, fixed, and stained with DAPI (Zha et al., 1996). The percentage of GFP-positive cells with fragmented or condensed nuclei (apoptotic) was determined (mean ± SD; n = 3).

cell line 293 (Zha et al., 1996). Consequently, pcDNA3-hBax was cotransfected with equal amounts of pcDNA3 parental vector (used as a negative control), pcDNA3-BI-1-HA (HA-tagged BI-1), or pRc/CMV-Bcl-2 (used as a positive control). One day later, both the floating and adherent cells were collected and subjected to the trypan-blue vital dye exclusion assay. BI-1 suppressed Bax-induced cell death in 293 cells to a similar extent as Bcl-2 (Figure 3A). DAPI staining of 293 cell nuclei confirmed that Bax-induced cell death occurred by apoptosis (not shown). Immunoblot analysis demonstrated that BI-1 does not interfere with Bax production in 293 cells (Figure 3B).

BI-1 Inhibits Apoptosis Induced by Growth Factor Deprivation, Etoposide, and Staurosporine, but Not Fas

The effects of BI-1 overexpression on apoptosis induced by a variety of stimuli were explored. For many of these experiments, we employed a green fluorescent protein (GFP)–tagged BI-1, because it provided a convenient marker for transfected cells and because BI-1-GFP accumulated to higher levels than the BI-1-HA protein (not shown). Transient transfection studies in 293 cells confirmed that the BI-1-GFP protein retained biological activity as a suppressor of Bax-induced cell death (Figure 3C).

In the human diploid fibroblast line GM701, ~30% of the cells transfected with control GFP-marker plasmid developed morphological characteristics typical of apoptosis when deprived of serum for ~30 hr. In contrast, GM701 cells transfected with plasmids encoding BI-1-GFP or Bcl-2 were substantially more resistant to serum deprivation, with only ~10% of the cells undergoing apoptosis (Figure 3D). Thus, BI-1 is able to suppress apoptosis induced by growth factor withdrawal in GM701 fibroblasts.

In many types of cells, apoptosis induced by the TNF-family receptor Fas (CD95) is poorly abrogated by Bcl-2 (reviewed by Vaux and Strasser, 1996). We therefore compared the effects of Bcl-2 with BI-1 in 293 cells where apoptosis was induced by transfection of Fas. Cotransfection of either BI-1 or Bcl-2 with a Fas-encoding plasmid failed to prevent apoptosis (Figure 3E). In contrast, Fas-induced cell death was markedly suppressed by cotransfection of a plasmid encoding XIAP, a protein that directly binds to and inhibits caspases required for Fas-induced apoptosis (Deveraux et al., 1997).

To further explore the role of BI-1 as an apoptosis inhibitor, we examined its effects in FL5.12, an interleukin-3-dependent pro-B lymphocyte clone previously shown to undergo apoptosis when deprived of IL-3. FL5.12 cells were stably transfected with plasmids encoding BI-1-GFP or Bcl-2 (as a positive control) and subclones obtained that expressed BI-1 or Bcl-2 at high levels. BI-1 transfectants were uniformly more resistant to apoptosis induction by IL-3 deprivation, with the extent of protection correlating roughly with the levels of

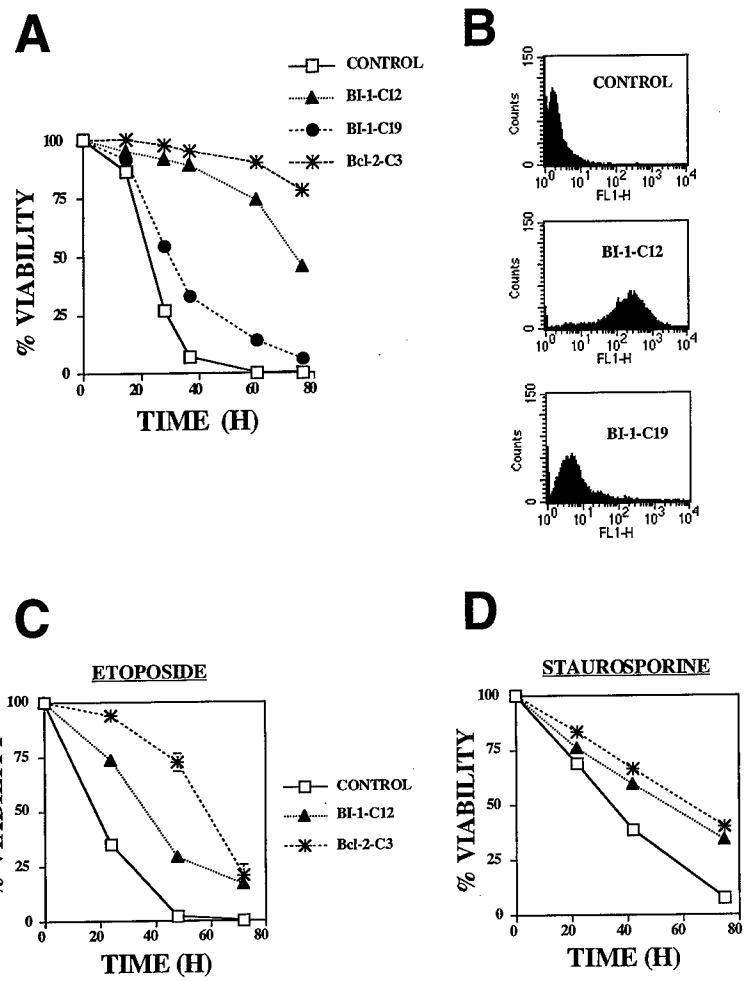

Figure 4. BI-1 Protects against Multiple Apoptotic Stimuli in FL5.12 Lymphocytes

(A) BI-1 inhibits IL-3 withdrawal-induced apoptosis in FL5.12 cells. Stably transfected cell lines expressing GFP-tagged BI-1 or untagged Bcl-2 were generated by electroporation. Independent clones were obtained by limiting-dilution for BI-1 (clone 12 and 19) and Bcl-2 (clone 3). Cells were grown to a density of $\sim 5 \times 10^5$ cells/ml before removing IL-3 from the medium. At various times thereafter, samples were removed and subjected to trypan blue dye exclusion assay (mean ± SD; n = 3).

(B) Expression of GFP-tagged BI-1 assessed by FACS analysis. Stably transfected FL5.12 cells containing either a negative control plasmid (pcDNA3) (top panel) or plasmid encoding BI-1-GFP were analyzed by FACS. The histograms are presented for clone 12 (middle panel), which expresses BI-1-GFP at high levels, as manifested by a single peak of green fluorescence at >2 logs above the negative control (top panel) and for clone 19 (bottom panel), which expresses BI-1-GFP at levels only ~0.5 log above background.

(C and D) FL5.12 cells were cultured with IL-3 and either 5 μg/ml etoposide (C) or 0.5 μM staurosporine. The percentage of viable cells was determined at various times thereafter by trypan blue dye exclusion assay (mean ± SD; n = 3). Some SD bars are obscured by symbols.

BI-1-GFP protein. Figure 4A presents results from two clones, one with only modest and another with high levels of BI-1-GFP protein production. Note that even clones with low levels of BI-1-GFP protein exhibited some resistance to IL-3 deprivation, whereas clones expressing high levels of BI-1-GFP protein displayed pronounced resistance to apoptosis induction by IL-3 withdrawal, with the prolongation in cell survival approaching that seen in Bcl-2-transfected FL5.12 cells. BI-1 overexpression also afforded protection in FL5.12 cells against apoptosis induced by etoposide and staurosporine. Comparisons of FL5.12 cell clones with various levels of BI-GFP expression suggested that BI-1 was generally somewhat less effective than Bcl-2 at conferring resistance against these agents (Figures 4C and 4D, and data not shown).

Taken together, the transfection experiments in mammalian cells (Figures 3 and 4) demonstrate that BI-1 has antiapoptotic activity in epithelial, fibroblastic, and hematopoietic cells, and suggest that BI-1 shares functional similarity with Bcl-2 in delaying cell death induced by Bax, growth factor deprivation, staurosporine, and etoposide, but not Fas.

BI-1 Antisense Induces Apoptosis

A BI-1 cDNA was subcloned into pcI-Neo in reversed orientation and transiently transfected into 293 cells, together with a GFP-marker plasmid. The BI-1 antisense (AS) plasmid induced apoptosis of 293 cells in a concentration-dependent manner, whereas control plasmid had no effect (Figure 5A). Lacking antibodies for detection of endogenous BI-1 protein to verify antisense-mediated down-regulation of BI-1 protein, parallel experiments were performed in which 293 cells were cotransfected with plasmids encoding BI-1-Flag protein and the BI-1-AS plasmid. As shown in Figure 5B, the levels of BI-1-Flag protein were markedly decreased in 293 cells that received the BI-1-AS plasmid compared to control transfected cells, as determined by immunoblotting. In contrast, the BI-1-AS plasmid had no effect on the levels of tubulin or other proteins examined, confirming the specificity of the results. These antisense experiments provide further evidence that BI-1 regulates apoptosis in mammalian cells.

BI-1 Is Located in Intracellular Membranes Similar to Bcl-2

The Bcl-2 and Bcl-X_L proteins are associated with intracellular membranes, primarily the outer mitochondrial membrane, endoplasmic reticulum, and nuclear envelope (Krajewski et al., 1993; González-Garcia et al., 1994), while Bax appears to be localized mostly to mitochondria (Zha et al., 1996). To preliminarily explore the

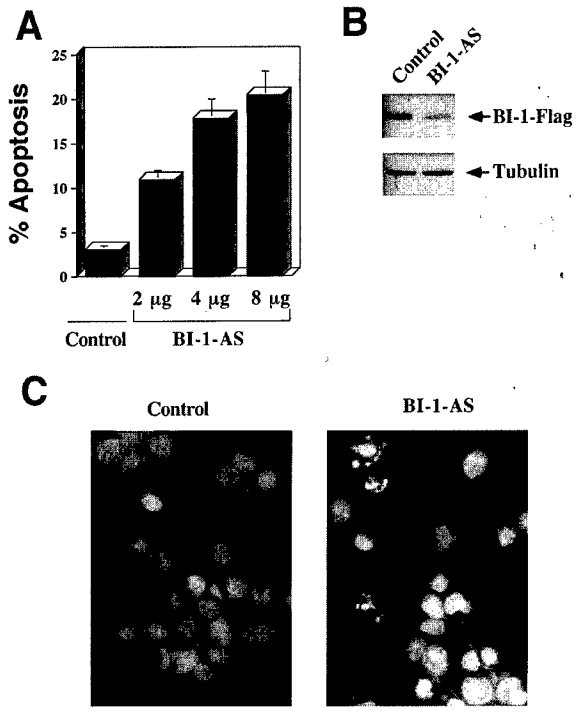

Figure 5. BI-1 Antisense Induces Apoptosis

(A) 293 cells were cotransfected with the indicated plasmids plus 1 μg of GFP-encoding plasmid. The total amount of plasmid used for each transfection was normalized at 9 μg. 30 hr after transfection, both the floating and adherent cells were pooled, fixed, and stained with DAPI. The percentage of apoptosis was determined as the ratio of cells with fragmented or condensed nuclei among GFP-positive cells (mean ± SD; n = 3). Data are representative of three independent experiments.

(B) 293 cells were cotransfected with 1 μg of BI-1-Flag-encoding plasmid in combination with either 8 μg of pcI-Neo or pcI-Neo-BI-1-AS. 30 hr after transfection, protein extracts were prepared, normalized for total protein content (30 μg per lane), and subjected to SDS–PAGE/immunoblot analysis using the anti-Flag M2 antibody for detection of BI-1-Flag protein and anti-tubulin antibody to control for loading.

(C) DAPI-stained cells as described in (A) were visualized and photographed under a UV microscope. Note at least four typical apoptotic cells with fragmented or condensed nuclei in the BI-1-AS-transfected population (right panel).

intracellular locations of the BI-1 protein, GFP-BI-1 protein was expressed in several different adherent cell lines (293, Cos-7, GM701). In all three cell lines, fluorescence microscopy demonstrated that BI-1 is exclusively cytosolic and appears to be associated with intracellular membranes in a pattern typical of the endoplasmic reticulum (ER) and its continuity with the nuclear envelope (Figure 6A and data not shown). In contrast, GFP control protein was diffusely distributed throughout the cells. Only a small portion of BI-1 appears to be associated with mitochondrial membranes, based on two-color analysis using a mitochondria-specific fluorescent dye (Figure 6B). Similar results were obtained using a Flag-tagged BI-1 protein instead of GFP-BI-1 (Figure 6Ac).

The intracellular location of BI-1 was also explored by subcellular fractionation experiments. For this purpose, 293T cells were transiently transfected with the BI-1-HA-encoding plasmid or vector control. Cells were lysed in hypotonic buffer 2 days later and separated into crude subcellular fractions of nuclei (N), heavy membranes (HM), light membranes (LM), and cytosol (C) by differential centrifugation as described (Wang et al., 1996). As shown in Figure 6C, BI-1 was found mostly in the HM and N fractions (inferred as nuclear envelope based on the GFP fusion localization studies), as determined by immunoblot analysis where the fractions were normalized for cell equivalents. A small proportion of the BI-1-HA protein was also found in the LM fraction. The HM fraction contains mitochondria, lysosomes, and rough ER, whereas the LM fraction contains smooth ER, endosomes, and plasma membranes. As a control for the fractionation procedure, the same blot was reprobed with antibodies specific for the mitochondrial inner membrane protein F_1-β-ATPase, the nuclear protein PARP, and the cytosolic protein CPP32 (caspase-3). Bcl-2 was found essentially in the same subcellular compartments as BI-1, with most of this protein associated with the HM and N fractions (Figure 6C). We conclude therefore that BI-1 is associated with intracellular membranes, based on GFP-tagging experiments, immunofluorescence microscopy, and subcellular fractionation studies.

Recently, it was reported that a subpopulation of Bax molecules in cells are not integrated into membranes, but rather are found within a soluble cytosolic fraction, with the relative proportion of membrane-associated Bax increasing after application of apoptotic stimuli (Hsu et al., 1997). Comparisons of BI-1- and control-transfected 293T cells, however, demonstrated that BI-1 does not substantially change the relative amounts of Bax protein associated with various subcellular compartments (Figure 6C).

BI-1 Associates with Bcl-2 In Vivo

The subcellular fractionation data suggest that BI-1 and Bcl-2 colocalize to the same intracellular membranes. To address the question of whether BI-1 and Bcl-2 physically associate in membranes, we performed in vivo cross-linking experiments. Plasmids encoding either Flag-tagged or HA-tagged BI-1 were cotransfected with Bcl-2 into 293 cells. Cells were then incubated 2 days later with the thiol-cleavable chemical cross-linker DTBP. As shown in Figure 7A, both the HA- and Flag-tagged BI-1 proteins could be cross-linked to Bcl-2, suggesting that BI-1 and Bcl-2 come within close proximity to each other in membranes. In contrast, Bax was not cross-linked to BI-1 (data not shown).

To further explore the interaction of BI-1 with Bcl-2 family proteins, we attempted to coimmunoprecipitate BI-1 with Bcl-2, Bcl-X_L, Bax, and Bak. For these experiments, 293 cells, which contain high levels of Bak but very little endogenous Bcl-2, Bcl-X_L, or Bax, were transfected with Bcl-2, Bcl-X_L, or Bax-expression plasmids and either Flag-tagged BI-1 plasmid or empty vector. As shown in Figure 7B, Flag-tagged BI-1 protein specifically coimmunoprecipitated with Bcl-2, and Bcl-X_L but not Bax or Bak. Testing of several deletion mutants of Bcl-2 revealed that the BH4 domain is required for interactions with BI-1 (not shown). This domain is uniquely found in antiapoptotic but not most proapoptotic members of the Bcl-2 family (Reed, 1997a), presumably explaining

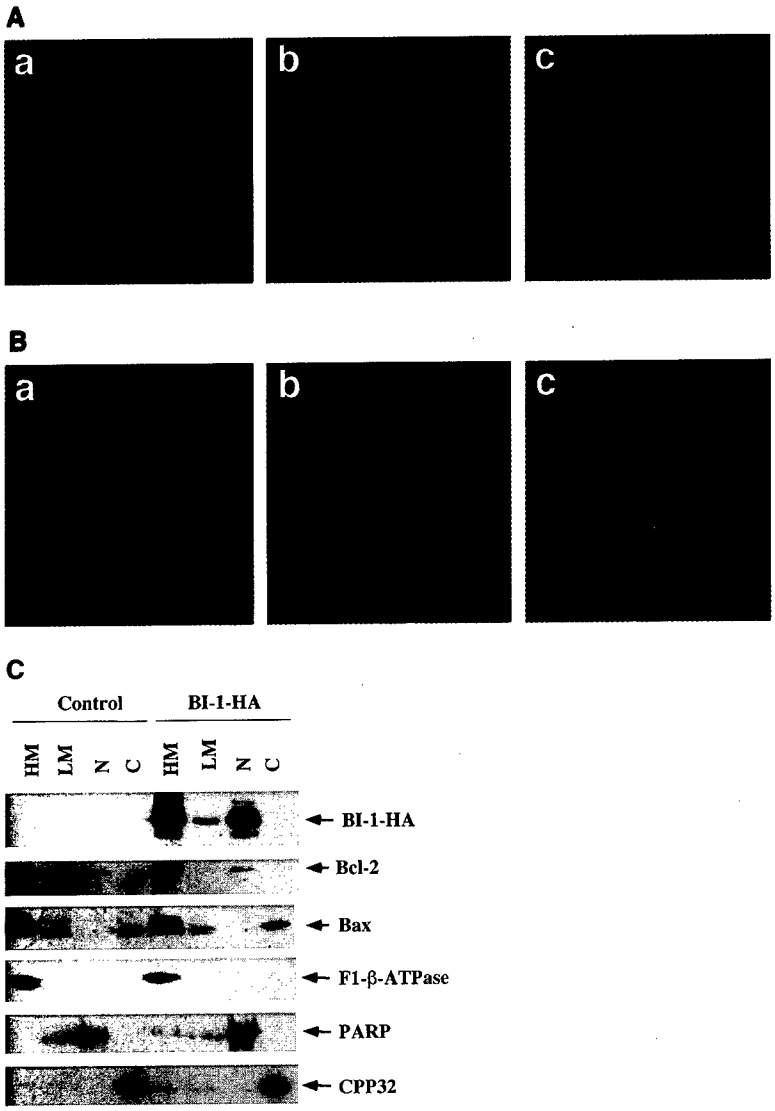

Figure 6. BI-1 Is Localized to Intracellular Membranes and Cofractionates with Bcl-2

(A) Either the parental pEGFP-N2 vector (a) or plasmid encoding BI-1-GFP fusion protein (b) or a plasmid encoding Flag-tagged BI-1 protein (c) was transfected into Cos-7 cells. 18 hr after transfection, cells were seeded in chamber slides for fluorescence microscopy. In (a) and (b), cells were analyzed directly using appropriate filters for visualization of the green fluorescence resulting from GFP. In (c), cells were stained with anti-Flag M2 and FITC-conjugated anti-mouse IgG. Cells stained with secondary antibody alone exhibited negligible fluorescence (not shown). Photographs represent ~400× original magnification.

(B) BI-1-GFP transfected Cos-7 cells were incubated with the Mitotracker dye before being fixed and visualized by fluorescence confocal microscopy using filters appropriate for the visualization of green (a), red (b), or both (c), resulting from the BI-1-GFP protein and the Mitotracker. Data shown are representative of the majority of doubly stained cells.

(C) 293T cells were transiently transfected with either parental vector (Control) or plasmid encoding HA-tagged BI-1. Cells were lysed 2 days later in a hypotonic solution, and crude subcellular fractionations were prepared. Equivalent proportions of each fraction were subjected to SDS–PAGE/immunoblot analysis using antibodies specific for HA-tag, Bcl-2, Bax, F1βATPase (mitochondria marker), PARP (nuclear marker), and CPP32 (Caspase-3, cytosolic marker).

why Bcl-2 and Bcl-X_L but not Bax and Bak form complexes with BI-1.

Discussion

By undertaking a functional screen for Bax suppressors in yeast, we have identified a novel human apoptosis inhibitor, BI-1. BI-1 is highly conserved throughout evolution. It shares no identifiable similarity to Bcl-2 family proteins or any other proteins implicated in PCD. Thus, BI-1 represents a novel type of apoptosis modulator. Interestingly, however, the predicted transmembrane topology and intracellular location of the BI-1 protein are somewhat similar to the presenilins, PS-1 and PS-2, which have been implicated in apoptosis and neurodegenerative disease, though no clear sequence homology is shared between BI-1 and these proteins (Wolozin et al., 1996; Guo et al., 1997).

Because BI-1 was identified by its ability to suppress Bax-induced yeast cell death, in theory, it could function upstream of, at the same level as, or downstream of Bax. For instance, BI-1 could act upstream of Bax, affecting the production of Bax or the targeting of Bax to its proper intracellular locations. Our data, however, suggest that BI-1 has no significant impact on the levels of Bax or its intracellular location. BI-1 does not appear to be associated with Bax, implying that it does not inhibit Bax directly. However, physical interaction is not necessarily required for mutual antagonism among anti- and proapoptotic Bcl-2 family proteins (Simonian et al., 1996; Tao et al., 1997; Zha and Reed, 1997). Given that the BI-1 associates with Bcl-2 and Bcl-X_L in mammalian cells, it is also formally possible that BI-1 functions through a Bcl-2 homolog in yeast. We do not favor this hypothesis, mainly because no Bcl-2 homologs are identifiable in *S. cerevisiae* on the basis of amino acid sequences deduced from the complete yeast genome.

BI-1 might function downstream of Bax, based on the apparent scarcity of BI-1 in mitochondrial membranes. In this regard, our preliminary attempts to localize BI-1 indicate that it is predominantly nonmitochondrial and instead may be mostly localized to the ER. In contrast,

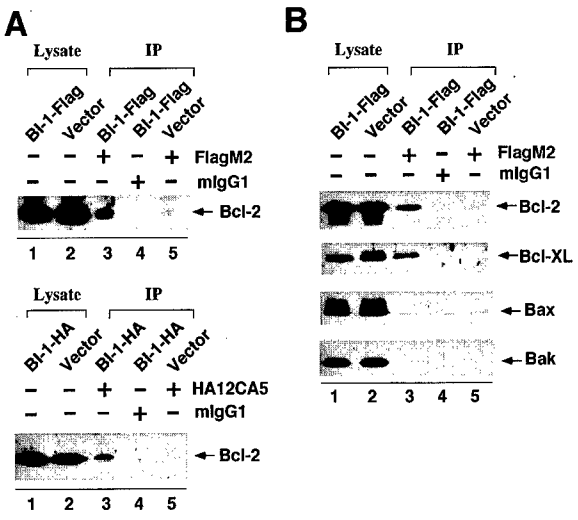

Figure 7. BI-1 Interacts with Bcl-2 in Mammalian Cells

(A) In vivo cross-linking. 293 cells were cotransfected with Bcl-2-encoding plasmid and either a control (vector) or plasmids encoding BI-1-Flag (top panel) or BI-1-HA (bottom panel) proteins. 2 days after transfection, cells were washed in PBS and incubated with the membrane-permeable chemical cross-linker DTBP. After cross-linking for 20 min, cells were washed in PBS and lysed in RIPA buffer. Immunoprecipitations were performed using normal mouse IgG1 as a negative control or the anti-Flag M2 (top) or anti-HA 12CA5 (bottom) monoclonal antibodies. Immunocomplexes were reduced (to reverse the cross-linking reaction) and analyzed by SDS–PAGE/immunoblotting using anti-Bcl-2 antiserum. Lanes 1 and 2 represent whole cell lysates from cells transfected with Bcl-2+BI-1 or Bcl-2+ vector, respectively (1/20 of the input for lanes 3–5). Lanes 3–5 were loaded with immune complexes precipitated with the indicated antibodies.

(B) Coimmunoprecipitation. 293 cells were transiently transfected with either Bcl-2- , Bcl-X_L-, or Bax-encoding plasmids together with either vector control plasmid or BI-1-Flag-encoding plasmid DNA. 2 days later, cells were lysed in 1% NP-40 buffer and immunoprecipitations were performed using either anti-Flag antibody M2 or IgG control. Immune complexes (lanes 3–5) were subjected to SDS–PAGE/immunoblot analysis using antisera specific for Bcl-2, Bcl-X_L, Bax, or Bak. Whole cell lysates (lanes 1 and 2) are from cells transfected with Bcl-2 (top), Bcl-X_L (second), Bax (third), or no plasmid (bottom) together with either BI-1-Flag plasmid (lane 1) or vector control (lane 2), representing 1/40 of the input for immunoprecipitation.

Bax has been reported to associate primarily with mitochondria in yeast and mammalian cells (Zha et al., 1996). Thus, to the extent that these two proteins reside in different organellar compartments, these observations support the notion of BI-1 operating downstream of Bax. However, others have shown that when Bcl-2 is targeted exclusively to the ER through substitution of the C terminus of cytochrome *b5*, it retains the ability to block apoptosis induced by some stimuli (Zhu et al., 1996). Moreover, the adenovirus Bcl-2 homolog E1B 19K can antagonize Bax-induced apoptosis but apparently is not associated with mitochondria (White and Cipriani, 1989; Han et al., 1996). This implies that Bcl-2 and E1B 19K need not necessarily be associated with mitochondria where Bax mostly resides, raising the possibility that Bcl-2 and Bax control parallel pathways that independently provide signals for cell survival and death, respectively. If true, then an alternative interpretation is that BI-1 is a downstream effector of Bcl-2, which prevents Bax-induced death in yeast by substituting for Bcl-2.

Though mitochondria have received much attention recently for their role in apoptosis, the ER has also been implicated in cell death regulation. The ER performs several essential functions, including protein processing and translocation, vesicle transport, and maintaining calcium homeostasis (reviewed by Teasdale and Jackson, 1996). It has been shown that Bcl-2 can alter regulation of Ca^{2+} in the ER, preventing loss of Ca^{2+} from this organelle following growth factor deprivation and decreasing the basal efflux of Ca^{2+} under normal circumstances (Baffy et al., 1993; Lam et al., 1994). Depletion of intra-ER Ca^{2+} stores has been reported to induce apoptosis (Baffy et al., 1993). Moreover, release of Ca^{2+} into the cytosol can induce mitochondrial permeability transition (PT) pore opening (Bernardi et al., 1994; Kroemer et al., 1996), thus creating a functional connection between the ER and mitochondria. Interestingly, antisense-mediated ablation of one of the ER Ca^{2+} channels, namely the inositol triphosphate–gated receptor-1, has revealed a requirement for this Ca^{2+} channel in T-cell apoptosis induced by diverse stimuli, including anti-Fas antibodies, glucocorticoids, and ionizing radiation (Jayaraman and Marks, 1997). Thus, regulation of Ca^{2+} trafficking through effects on Ca^{2+} channels in the ER represents one of several tenable hypotheses for explaining how a multiple-membrane-spanning protein such as BI-1 might directly or indirectly modulate cell death pathways.

What might BI-1 be doing in intracellular membranes? Based on the predicted multiple transmembrane segments, BI-1 could possibly function as a receptor or an ion-channel protein. Similar to ion-channels, some of the putative transmembrane segments of BI-1 when examined on α-helical wheel plots predict the presence of hydrophilic residues on one face of the α helices rather than uniformly hydrophobic residues like those found in the transmembrane domains of most cell surface growth factor receptors. If several amphipathic helices assembled in the membrane, creating an aqueous lumen ringed by the hydrophilic surfaces of these putative α helices, then BI-1 could potentially form an ion channel in membranes. Alternatively, the apparent physical association of BI-1 and Bcl-2 raises the intriguing possibility that BI-1 and Bcl-2 could create heteromeric channels, with Bcl-2 presumably contributing its 5th and 6th amphipathic α-helical domains, which have been shown to be required for in vitro pore formation (Schendel et al., 1997), and BI-1 providing some of its amphipathic transmembrane α-helical segments. Precedent for this idea exists among some types of K^+ channels in which the functional channel is comprised of a heterotetramer, with each subunit contributing transmembrane α helices that surround a central ion pore (Catterall, 1995). Alternatively, the interaction of Bcl-2 and BI-1 could provide a mechanism by which BI-1 regulates the previously described Bcl-2 channel, or conversely, by which Bcl-2 regulates a hypothetical BI-1 channel, analogous to some voltage-gated Na^+ and Ca^{2+} ion channels in which a single protein forms the actual channel but this channel is highly regulated by associated integral

membrane proteins (Catterall, 1995). Also, by functioning as an ion channel, conceivably BI-1 might alter ion gradients, pH, or voltage (DV) across the membranes where Bcl-2 family proteins reside, thus either inhibiting or enhancing their ability to integrate into membranes and form ion channels (review by Reed, 1997a). It should be noted however that Bcl-2 has been reported to bind multiple proteins (Reed, 1997a) and thus may be promiscuous in its interactions with other proteins, including BI-1. Thus, while the significance of the interaction of BI-1 with Bcl-2 remains to be determined, the data presented here nevertheless establish that BI-1 is a novel suppressor of cell death.

The yeast-based functional cloning strategy employed here provides an alternative approach for identifying proteins that regulate mammalian cell death. Though BI-1 can interact with Bcl-2 (or with a complex of proteins that includes Bcl-2), it is unlikely that interaction cloning methods based on either yeast two-hybrid or λ-phage expression library screening using ligand-blotting would have detected BI-1, because of its hydrophobic characteristics. Protein purification attempts based on ability to interact with Bcl-2 also would have been unlikely to succeed for the same reason. Thus, functional screening for Bax suppressors in yeast provides a powerful approach that complements these other methods and that seems likely to yield new insights into the biochemistry and genetics of mammalian cell death regulation. Future studies, including targeted gene knockout experiments in mice, will better define the overall role played by BI-1 in the Bcl-2/Bax pathway for cell death regulation.

Experimental Procedures

Yeast Methods

Yeast strains used included: BF264-15Dau (*MATa ade1 his2 leu2-3, 112 trp1-1a ura3*) (Lew et al., 1991) and EGY48 (*MATα trp1 ura3 his3 leu2::plexAop$_6$-LEU2*. Strain QX95001 is BF264-15Dau containing the *LEU2*-marked mBax-encoding plasmid YEp51-Bax. Strains BF264-15Dau and EGY48 were maintained in the rich YPD medium, and strain QX95001 was maintained in SD-Leu (synthetic dropout medium lacking leucine). Transformations, plasmid extractions, and protein extracts were prepared as described (Sato et al., 1994; Zha et al., 1996).

For cDNA library screening, QX95001 cells were grown to mid-log phase in the SD-Leu liquid medium and transformed with 100 μg of HepG2 cDNA library DNA by a LiOAc method. Bax-resistant transformants were directly selected on galactose-containing synthetic dropout medium lacking leucine and uracil (SD-leu+ura). An aliquot of transformation mixture was also spread on glucose-containing medium to determine transformation efficiency. Bax-resistant colonies were patched onto galactose-containing SD-leu+ura plates. Con-loss assays were performed as described (Ausubel et al., 1991).

Plasmid Constructions

pcDNA3-hBax and pRc/CMV-Bcl-2 contain human Bax and human Bcl-2 cDNAs in the expression plasmids pcDNA3 and pRc/CMV, respectively. The C-terminal HA-tagged BI-1 plasmid was constructed in two steps. The C terminus of BI-1 (from the internal BamHI site) was first PCR-amplified to add a XhoI site to the very C terminus of the BI-1 ORF (just before the stop coden). PCR primers used were 5′-GGGGATCCATTTGGCCTTTCCAG and 5′-GGCTCGA GTTTTCTTCTCTTTCTTCTTATCC. The resulting PCR product was digested with BamHI and XhoI and subcloned into pcDNA3 containing an oligonucleotide encoding three in-frame copies of the HA tag downstream of the XhoI site, producing plasmid pQX9645. Next, the N-terminal portion of BI-1 was re-ligated into pQX9645, giving rise to pcDNA3-BI-1-HA. An EcoRI–XhoI fragment containing BI-1 cDNA from pcDNA3-BI-1-HA was subcloned into pcDNA3 containing a single copy of the FLAG epitope downstream of the XhoI site and into the GFP vector pEGFP-N2 (Clontech Inc.) between the EcoRI and SalI sites. The 2.6 kbp BI-1 cDNA was subcloned in reverse (antisense) orientation between the XhoI and EcoRI sites of pcI-Neo (Invitrogen, Inc.), producing pcI-Neo-BI-1-AS.

Cell Culture and Transfections

293, 293T, GM701, and Cos7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (fetal bovine serum), 1 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate and transfected as described (Zha et al., 1996). For cell death assays, 293 cells were seeded at 8×10^5 cells per 60 mm dish and 1 day later transfected with various combinations of plasmids (9 μg total DNA). Precipitates were removed after 8 hr and replaced with fresh medium. Both floating and adherent cells (after trypsinization) were collected at 24 hr post-transfection and analyzed by trypan blue dye exclusion assay, counting a minimum of 300 cells and performing experiments in triplicate (mean value ± SD). The transfection efficiency was estimated to be ≥ 70% based on cotransfections with a β-gal reporter plasmid. DAPI staining for assessing nuclear morphology was also performed.

For GM701 fibroblasts, cells were seeded into 6-well tissue culture dishes at 6×10^4 cells/well and cotransfected with 0.5 μg of β-gal reporter plasmid and 4 μg of various other expression plasmids. Precipitates were removed 6 hr later, and 18 hr posttransfection cells were washed three times with DMEM and incubated in medium containing 0.1% FBS for 30 hr before fixing and staining with X-gal (Zha et al., 1996).

FL5.12 cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 10% WEHI-3B conditioned medium, L-glutamine, and antibiotics. Plasmid DNAs (20 μg) were introduced into FL5.12 cells by electroporation (GenePulser; Biorad) using 650 V/cm and 1025 μF, followed by selection in medium containing puromycin (0.5 μg/ml). Clones expressing BI-1-GFP fusion protein were screened initially by fluorescence microscopy, followed by FACS and immunoblot analysis. IL-3 withdrawal and cell viability determinations were performed as described (Wang et al., 1995). For drug-resistance assays, either etoposide (5 μg/ml) or staurosporine (0.5 μM) was added to cells at a density of 5×10^5 cells/ml and incubated for up to 3 days before determining the percentage cell viability based on trypan blue dye exclusion.

Immunoprecipitation and Immunoblot Assays

Cell lysates were prepared using HKME solution (10 mM HEPES [pH 7.2], 142 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA) containing 1% NP-40 and protease inhibitors (1 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 0.7 μg/ml pepstatin) and cleared by incubation with the rec–protein G–Sepharose 4B (ZYMED). Lysates were then diluted in HKME to a final concentration of 0.6% NP-40 and incubated with anti-Flag M2 antibody (Kodak) at 4°C for 2 hr with constant rotating, followed by rec–protein G beads for 1 hr. Immobilized immunocomplexes were washed four times in HKME containing 0.2% NP-40 before boiling in SDS sample buffer. Cell lysates or immunoprecipitates were resolved by SDS–PAGE and transferred onto nitrocellulose membranes (Biorad). After blocking with 5% non-fat milk, 2% bovine serum albumin (BSA) in TBST (10 mM Tris [pH 7.5]; 142 mM NaCl; 0.1% Tween 20) at the room temperature for 2 hr, blots were incubated in the same solution with various primary antibodies, including monoclonal antibodies specific for HA (12 CA5 [0.8 μg/ml]) or Flag (M2 [3 μg/ml]) and polyclonal antisera specific for Bcl-2 (0.1% [v/v]), Bcl-X_L (0.1%), Bax (0.1%), CPP32 (0.1%), PARP (0.1%), or F_1-β-ATPase (0.1%), followed by 0.6 μg/ml horseradish peroxidase–conjugated anti-mouse or anti-rabbit IgG (Biorad) secondary antibodies. Bound antibodies were visualized using an enhanced chemiluminescence (ECL) detection system (Amersham).

In Vivo Cross-Linking

Transfected 293 cells were washed twice with PBS and then incubated for 20 min at room temperature in PBS with 1 mM DTBP

(dimethyl-3,3′-dithiobispropionimidate [2HCl]) (Pierce, Inc.) with gentle shaking. After extensive washing in PBS, cells were lysed in RIPA (10 mM Tris [pH 7.4], 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS) containing protease inhibitors, and immunoprecipitations were performed using anti-HA (12CA5) or anti-Flag (M2) monoclonals. Immune complexes were immobilized on protein G beads and washed four times with RIPA, followed by boiling in SDS sample buffer (containing 5% β-mecaptoethanol). Samples were analyzed by SDS–PAGE/immunoblotting using rabbit antisera specific for Bcl-2 or Bax.

Immunofluorescence and Confocal Microscopy

Cells transiently transfected with pcDNA3-BI-1-Flag were trypsinized and seeded into chamber slides. The next day, cells were washed in PBS and fixed in PBS containing 3.7% paraformaldehyde, followed by washing twice in PBS. Cells were then permeabilized in 0.1% Triton X-100/PBS for 20 min and preblocked in PBS containing 3% BSA, 2% FBS, 0.1% goat serum. Anti-Flag antibody M2 (3 μg/ml) was added to cells in the same solution and incubated for 1 hr, followed by washing three times in PBS/0.1% Triton X-100 and incubation with 2 μg/ml FITC-conjugated secondary anti-mouse antibody (Dako) for 1 hr. After washing three times in PBS, slides were covered in Vectashield mounting medium (Vector Laboratories, Inc.) and sealed with nail polish. For two-color analysis, GFP-BI-1-transfected cells were incubated with 20 nM Mitotracker (Molecular Probes, Inc.) in normal growth medium for 20 min at 37°C. Confocal fluorescence microscopy was performed using an Axiophot photomicroscope (Zeiss, Inc., Oberkochen, Germany).

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The Mitochondrial F_0F_1-ATPase Proton Pump Is Required for Function of the Proapoptotic Protein Bax in Yeast and Mammalian Cells

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Summary

The proapoptotic mammalian protein Bax associates with mitochondrial membranes and confers a lethal phenotype when expressed in yeast. By generating Bax-resistant mutant yeast and using classical complementation cloning methods, subunits of the mitochondrial F_0F_1-ATPase proton pump were determined to be critical for Bax-mediated killing in *S. cerevisiae*. A pharmacological inhibitor of the proton pump, oligomycin, also partially abrogated the cytotoxic actions of Bax in yeast. In mammalian cells, oligomycin also inhibited Bax-induced apoptosis and activation of cell death proteases. The findings imply that an intact F_0F_1-ATPase in the inner membrane of mitochondria is necessary for optimal function of Bax in both yeast and mammalian cells.

Introduction

Altered function of mitochondria has been recognized for many years as an important contributor to ischemic and necrotic cell death (Bernardi et al., 1994). Recently, however, evidence has accumulated suggesting a critical role for these organelles in apoptosis and programmed cell death (Petit et al., 1996; Hirsch et al., 1997).

Proteins of the Bcl-2 family are important regulators of mammalian cell life and death, with some functioning to prevent and others to promote apoptosis (Reed, 1994; Yang and Korsmeyer, 1996). These proteins can also modulate cell death processes that result in necrotic rather than apoptotic cell death, under some circumstances (Kane et al., 1995; Shimizu et al., 1996). Most Bcl-2 family proteins are integral membrane proteins that reside in the outer mitochondrial membrane, as well as some other intracellular membranes (Krajewski et al., 1993; González-Garcia et al., 1994).

At present, the biochemical mechanism by which Bcl-2 and its homologs regulate cell death remains controversial (Reed, 1997). The three-dimensional structure of one of the Bcl-2 family proteins suggests similarity to the pore-forming domains of certain bacterial toxins, such as diphtheria toxin and the colicins (Muchmore et al., 1996). Moreover, the antiapoptotic proteins Bcl-2 and Bcl-X_L as well as the proapoptotic protein Bax can form ion channels in synthetic membranes in vitro in a pH-dependent manner (Antonsson et al., 1997; Minn et al., 1997; Schendel et al., 1997; Schlesinger et al., 1997). However, Bcl-2 and Bcl-X_L also clearly have apoptosis-regulatory functions apart from their ability to form channels, in that they bind to several other proteins that can modulate responses to apoptotic stimuli (Reed, 1997). Bcl-2 has been shown to protect mitochondria from loss of membrane potential and release of caspase-activating proteins such as cytochrome c and apoptosis-inducing factor (AIF) (Susin et al., 1996; Zamzami et al., 1996; Kluck et al., 1997; Yang et al., 1997), whereas Bax can induce loss of mitochondrial membrane potential and activation of caspases (Xiang et al., 1996; Jürgensmeier et al., 1997). It remains unclear whether these effects of Bcl-2 and Bax on mitochondrial physiology are a direct result of their intrinsic activities as channel proteins, which conceivably may transport either ions or proteins, as opposed to an indirect consequence of their effects on other channel proteins associated with mitochondrial membranes.

When expressed in either the budding yeast *Saccharomyces cerevisiae* or the fission yeast *Schizosaccharomyces pombe*, the proapoptotic mammalian protein Bax confers a lethal phenotype (Sato et al., 1994; Ink et al., 1997; Jürgensmeier et al., 1997). Yeast cells undergoing Bax-induced death exhibit ultrastructural changes that include massive cytosolic vacuolarization and apparent disruption of mitochondria (Ink et al., 1997; Jürgensmeier et al., 1997), similar to mammalian cells that express Bax in the presence of caspase inhibitors (Xiang et al., 1996). In yeast, the Bax protein is associated primarily with mitochondria, and the targeting of Bax to these organelles appears to be important for its lethal phenotype in yeast (Zha et al., 1996). Similar to mammalian cell apoptosis, expression of Bax in yeast has been reported to induce release of cytochrome c from mitochondria into the cytosol (Manon et al., 1997). Yeast cell death induced by Bax or the closely related Bak protein can be specifically suppressed by antiapoptotic Bcl-2 family proteins (Sato et al., 1994; Bodrug et al., 1995; Hanada et al., 1995; Greenhalf et al., 1996; Zha et al., 1996; Ink et al., 1997; Jürgensmeier et al., 1997). Further evidence of similarities in at least some of the mechanisms by which Bax functions in animal cells and yeast comes from the observation that cell death induced by Bax can be suppressed in both mammalian cells and *S. cerevisiae* by BI-1, a human protein that contains multiple membrane-spanning domains (Xu and Reed, 1998 [this issue of *Molecular Cell*]).

The functions of Bcl-2 family proteins are often conserved across evolution, with the human Bcl-2 protein, for example, exhibiting potent antiapoptotic activity even in nematodes and insect cells (Alnemri et al., 1992; Vaux et al., 1992; Hengartner and Horvitz, 1994). Moreover, the human Bcl-2 protein has been reported to protect superoxide dismutase (sod)–deficient strains of budding yeast from cell death induced by oxidative stress (Kane et al., 1993), implying an evolutionarily

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conserved function perhaps even in some unicellular organisms. Though programmed cell death (PCD) is conventionally thought to operate only in multicellular organisms, recent studies have revealed apoptosis-like cell death in several unicellular eukaryotes, including *Dictyostelium discoideum* (Cornillon et al., 1994), *Trypanosome brucei rhodesiense* (Welburn et al., 1996), *Trypanosome cruzi* (Ameisen et al., 1996), *Leishmania amazonensis* (Moreira et al., 1996), and *Tetrahymena thermophila* (Christensen et al., 1995). It has also been suggested that certain forms of PCD may even exist in prokaryotes (Ameisen, 1996), where cell suicide mechanisms could potentially limit spread of viruses, reduce competition for nutrients during times of starvation, or ensure that cells with damaged DNA do not pass their defective genomes on to future generations. Reasoning that at least some of the functions of Bcl-2 family proteins appear to be conserved in yeast, we undertook a classical genetics approach designed to identify yeast genes that are required for Bax-mediated lethality in *S. cerevisiae*.

Results

Creation of a Mutant Yeast Strain that Displays Resistance to Bax-Induced Cell Death

Yeast strain EGY48 was mutagenized with N-methyl-N′-nitro-N-nitrosoguanidine (MMNG) and then transformed with YEp51-Bax, a *LEU2*-marked, high-copy episomal plasmid that produces the mouse Bax protein under the control of the galactose-inducible *GAL10* promoter (Zha et al., 1996). Transformants were plated on leucine-deficient semisolid medium containing galactose, resulting in ~50 viable colonies. To exclude clones that might have survived because of defects in transactivation of the *GAL10* promoter in YEp51-Bax, these transformants were cured of the YEp51-Bax plasmid and then retransformed with the plasmid pEG202-Bax in which Bax is produced from a strong constitutive *ADH1* promoter. The resulting 24 Bax-resistant clones were mated with wild-type Myy290 strain cells, yielding 9 diploids in which sensitivity to Bax-mediated killing was restored, thus suggesting a recessive mutation. Tetrad analysis was then performed for these recessive mutants, with only one (hereafter designated as Bax-resistant mutant-1 [BRM1]) exhibiting 2:2 Mendelian segregation of the Bax-resistance phenotype in a manner consistent with a single gene defect (Figure 1A).

The genomic mutation in BRM1 cells did not interfere with Bax protein production, as determined by immunoblotting (Figure 1B). For these experiments, the wild-type and BRM1 cells were transformed with pGilda-Bax, which produces Bax as a fusion protein with a portion of LexA (used analogous to an epitope tag here), or the pGilda plasmid, which produces only the LexA fragment. The addition of the LexA tag to Bax does not interfere with its intracellular targeting or cell death–inducing function in yeast (Zha et al., 1996).

Complementation Cloning of Yeast Genes that Restore Sensitivity to Bax

The BRM1 cells containing pGilda-Bax were transformed with a centromere-based yeast genomic library.

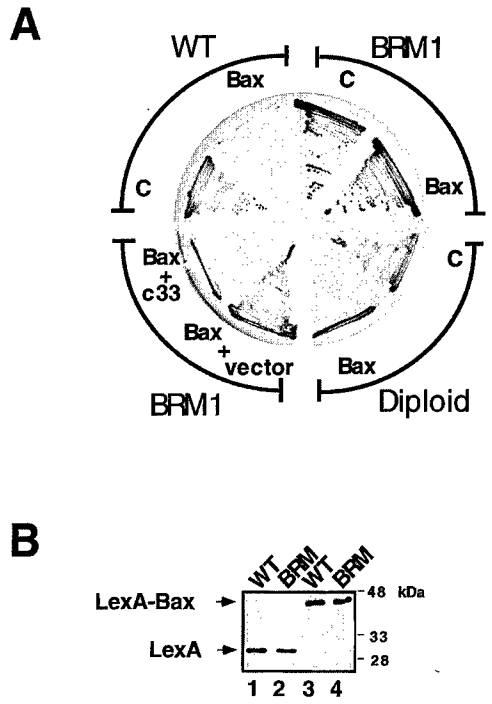

Figure 1. Generation of a Bax-Resistant Yeast Mutant and Identification of Genomic Clones that Restore Bax Sensitivity

(A) Wild-type EGY48 yeast (WT), Bax-resistant-mutant (BRM1), and diploid cells derived from mating Myy290 (wild-type) × BRM1 were transformed with control plasmid pGilda (C) or galactose-inducible pGilda-Bax (Bax). BRM1 was also cotransformed with pGilda-Bax and clone 33 from yeast genomic library or control vector YCp50. Each transformant was first grown on glucose-based plate and then a colony was restreaked on galactose-containing plates and incubated at 30°C for 4 days.

(B) Immunoblot analysis is shown for lysates (10 μg) derived from WT yeast (lanes 1 and 3) and BRM1 (lanes 2 and 4) cells transformed with pGilda (produces LexA protein DNA-binding domain without a nuclear localization sequence) (lanes 1 and 2) or pGilda-Bax (produces LexA-Bax fusion protein) (lanes 3 and 4). Antigens were detected using anti-LexA rabbit antiserum.

Eight transformants were identified by replica plating that appeared to have a restoration of their sensitivity to Bax-mediated cell death. The plasmids recovered from these eight transformants were then retransformed with pGilda-Bax into BRM1 cells, with only one of these clearly restoring sensitivity to Bax-mediated lethality to wild-type levels (Figure 1A). DNA sequence analysis revealed that this clone contained three genes, including *ATP4*, which encodes subunit 4 of the yeast F_0F_1-ATPase, a proton pump located in the inner membrane of mitochondria (Weber and Senior, 1997).

ATP4 Is Required for Bax-Induced Lethality in Yeast

Since it has been previously suspected that Bax promotes cell death at least in part through effects on mitochondria (Xiang et al., 1996; Zha et al., 1996), we focused on *ATP4* as a likely candidate gene that is required for Bax-induced killing of yeast. The *ATP4* gene has been previously disrupted in *S. cerevisiae* by *URA3* insertional mutagenesis (Velours et al., 1989; Paul et al., 1992), thus

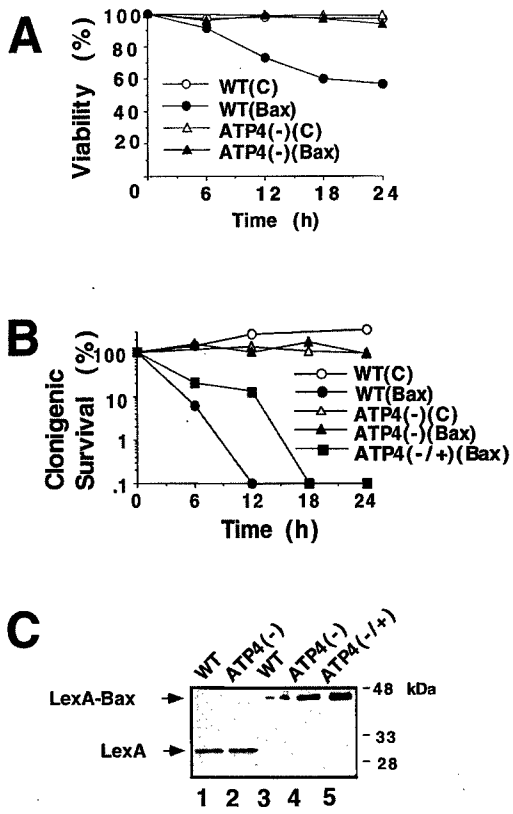

Figure 2. Yeast with Disrupted *ATP4* Gene Are Resistant to Bax-Induced Cell Death

Wild-type D273-10B/A yeast, *ATP4* knockout yeast strain PVY10 (*ATP4*$^-$), and heterozygous diploids derived from mating of PVY10 with Myy290 (*ATP4*$^{-/+}$) were transformed with pGilda (C) or pGilda-Bax. The cells were grown in glucose-based medium and then recultured in galactose-based medium to induce protein expression from the *GAL1* promoter in pGilda plasmids.

(A) The percentage of trypan blue dye excluding cells was determined at various times after switching to galactose-based media (mean ± SE; n = 3; SE bars are obscured by symbols).

(B) Clonigenic survival was determined by recovering cells at various times from galactose-containing medium and plating 1000 cells on glucose-based semisolid medium. Data are representative of at least three experiments.

(C) Immunoblot analysis was performed to assess LexA and LexA-Bax protein levels in cells after 12 hr of culture in galactose-based media, as described for Figure 1.

creating the *ATP4*-deficient strain PVY10. We therefore tested PVY10 cells for resistance to Bax. For these experiments, PVY10 cells were transformed with pGilda-Bax or pGilda control plasmid and grown for various times in galactose-containing medium to induce expression of Bax, and cell viability was monitored by trypan blue dye exclusion. *ATP4*-deficient PVY10 cells were not killed after switching from glucose- to galactose-containing medium (Figure 2A). In contrast, cells of the isogenic wild-type strain (D273-10B/A) that had been transformed with pGilda-Bax began to die within 12 hr after switching to galactose.

To further verify that *ATP4*-deficient PVY10 cells are resistant to Bax-mediated lethality, a clonigenic survival assay was performed in which cells were switched from glucose- to galactose-containing liquid medium for 1 day to induce expression of Bax, and then plated on glucose-based semisolid medium, which suppresses the *GAL1* promoter in pGilda. The plating efficiency of *ATP4*-deficient PVY10 cells was essentially the same, regardless of whether they contained the pGilda-Bax or pGilda plasmids (Figure 2B). In contrast, colony formation by the isogenic wild-type strain was markedly reduced in cells harboring the pGilda-Bax plasmid compared to the control pGilda vector. Clonigenic survival of the wild-type cells began to decline after as little as 6 hr of exposure to galactose, with essentially all cells failing to form viable colonies after a 12 hr exposure. The differences in the kinetics of loss of clonigenic survival and development of trypan blue positivity (compare Figures 2A and 2B) have been observed previously in Bax-expressing yeast (Zha et al., 1996; Jürgensmeier et al., 1997), and likely reflect a commitment to cell death even before loss of plasma membrane integrity. Mating the PVY10 cells with wild-type haploids to create *ATP4*$^{+/-}$ heterozygous diploids restored sensitivity to Bax. Immunoblot analysis demonstrated that the *ATP4* mutation did not prevent production of Bax protein (Figure 2B). We conclude therefore that *ATP4*, which is a nuclear gene that encodes subunit 4 of the yeast mitochondrial F_0F_1-ATPase proton pump, is required for Bax-mediated killing of yeast.

The Proton-Pump Inhibitor Oligomycin Inhibits Bax-Induced Killing of Yeast

Oligomycin binds to the F_0 portion of the yeast and mammalian F_0F_1-ATPases and prevents the proton pump from transporting H^+ ions, thus effectively shutting it off (Tzagoloff, 1970). We reasoned that if the proton pump is required for Bax-mediated lethality in yeast, oligomycin should render wild-type yeast resistant to Bax. To test this hypothesis, D273-10B/A strain yeast that had been transformed with either pGilda-Bax or pGilda control plasmids were cultured for various times in galactose-containing medium with or without oligomycin. Oligomycin did not inhibit the growth of yeast under these conditions due to their ability to produce sufficient ATP from anaerobic fermentation (not shown). Oligomycin is nontoxic in yeast and it fails to induce permeability transition pore (PTP) opening in yeast mitochondria (Jung et al., 1997). As shown in Figure 3, oligomycin partially inhibited the Bax-induced killing of yeast, without interfering with production of the LexA-Bax protein. Thus, similar to disruption of the *ATP4* gene, a pharmacological inhibitor of the F_0F_1-ATPase proton pump suppresses Bax-mediated lethality in yeast.

Respiration Is Not Required for Bax-Induced Killing of Yeast

It was possible that the genetic disruption or pharmacological inhibition of the F_0F_1-ATPase proton pump indirectly suppressed Bax-mediated lethality in yeast by blocking respiration (Velours et al., 1989; Paul et al., 1992; Giraud and Velours, 1994). To address this question and to further explore the importance of the F_0F_1-ATPase, we compared the effects of Bax expression on the viability and clonigenic survival of *rho*$^-$ yeast as well as on an additional mutant strain of yeast in which the

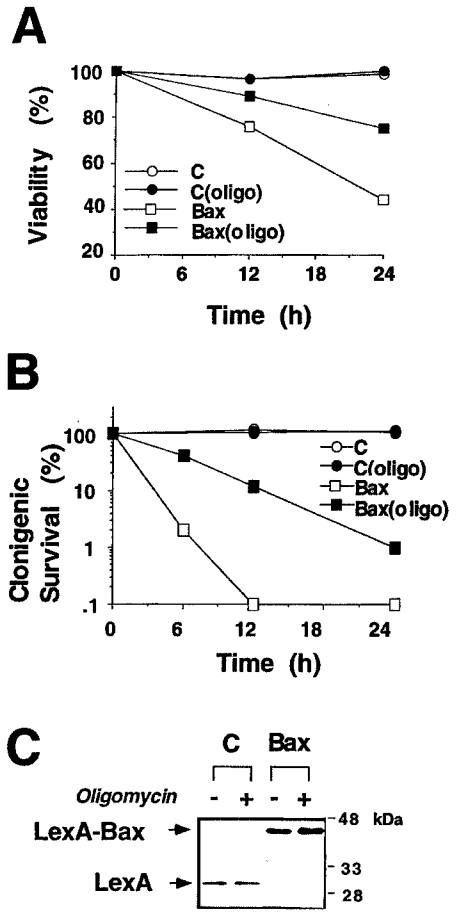

Figure 3. F_0F_1-ATPase Proton-Pump Inhibitor, Oligomycin, Attenuates Bax-Induced Cell Death in Yeast

Yeast strain D273-10B/A cells transformed with pGilda (C) or pGilda-Bax were initially cultured in glucose-based media, then switched to galactose-containing medium with (closed symbols) or without (open symbols) 10 μM oligomycin, and the percentage of trypan blue dye excluding cells was determined at various times thereafter (A) or cells were recovered and either 1000 or 3000 cells were plated on glucose-containing semisolid medium (B) (mean ± SE; n = 3; SE symbols are obscured by symbols). In (C), protein lysates (10 μg) were generated from the same cells after 12 hr of culture in galactose-based medium and analyzed by immunoblotting using anti-LexA antiserum. Lanes 1/2 and lanes 3/4 represent cells containing pGilda or pGilda-Bax, respectively, grown with or without oligomycin as indicated.

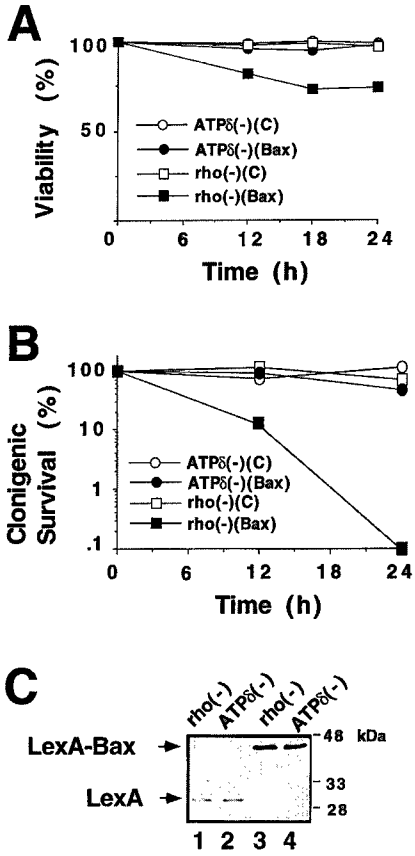

Figure 4. *ATP*δ-Deficient but Not *rho*− *petite* Yeast Are Bax-Resistant

Strain CGY1 yeast that harbor a *URA3*-disrupted *ATP*δ gene and *rho*⁻ yeast (both *petite*) were transformed with pGilda ([C]; open symbols) or pGilda-Bax (closed symbols). Cell viability (A), clonigenic survival (B), and Bax protein levels (C) were measured as described for Figure 2 at various times after switching cells to galactose-containing medium (mean ± SE; n = 3; some SE symbols are obscured by symbols). In (C), lanes 1/2 and lanes 3/4 represent *rho*⁻ and *ATP*δ-deficient yeast transformed with pGilda and pGilda-Bax, respectively.

δ subunit of F_0F_1-ATPase had been inactivated by *URA3* insertional mutagenesis (Giraud and Velours, 1994). Unlike the *ATP4* mutant, both *rho*⁻ and *ATP*-δ-deficient yeast are *petites*. *Rho*⁻ yeast fail to express all proteins encoded in the mitochondrial genome, and thus lack certain proteins that are critical for respiration. Though certain subchains of the F_0F_1-ATPase proton pump are encoded in the mitochondrial genome, its activity remains partially functional in *rho*⁻ yeast (Schatz, 1968; Giraud and Velours, 1994). In contrast, loss of the nuclear-encoded *ATP*-δ protein results in deficient function of both the F_0 and F_1 components of the proton pump (Giraud and Velours, 1997). These two *petite* strains grew at comparable rates in the absence of Bax (not shown).

The *rho*⁻ yeast transformed with pGilda-Bax began to die when switched to galactose-containing medium, whereas *rho*⁻ cells containing the control pGilda vector did not (Figure 4A). Note however that the Bax-induced cell death and loss of clonigenic survival occurred with delayed kinetics relative to wild-type yeast (compare with Figure 2). Thus, the absence of respiration in *rho*⁻ cells may reduce but does not abrogate Bax-mediated lethality in yeast. In contrast, yeast lacking the δ subunit of the F_0F_1-ATPase were completely resistant to Bax, despite expressing LexA-Bax protein at levels equivalent to those of the *rho*⁻ cells (Figure 4). We conclude therefore that respiration is not required for Bax-induced killing of yeast, but the F_0F_1-ATPase proton pump is.

Oligomycin Also Inhibits Bax-Induced Apoptosis and Activation of Cell Death Proteases in Mammalian Cells

No mammalian cells exist that harbor mutations within subunits of the mitochondrial F_0F_1-ATPase. Thus, to explore whether the proton pump is also required for optimal function of Bax in mammalian cells, one is limited

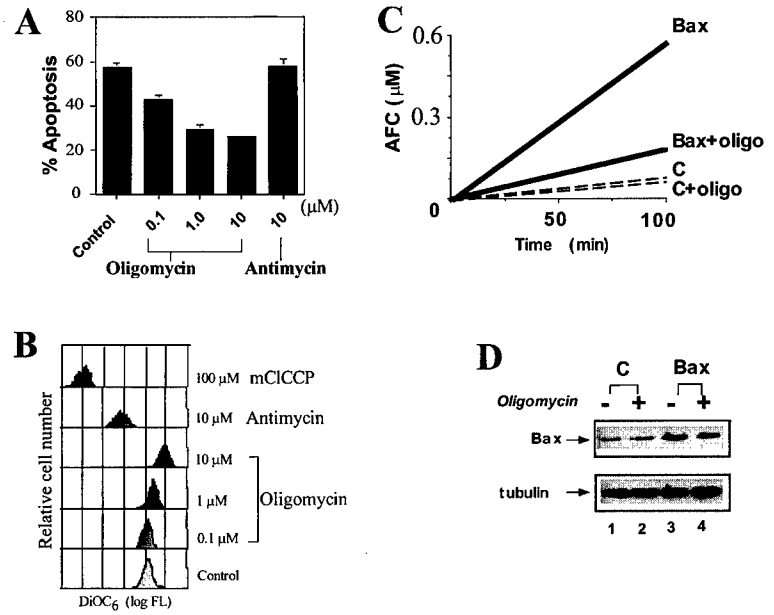

Figure 5. The F_0F_1-ATPase Inhibitor Oligomycin Suppresses Bax-Induced Apoptosis and Caspase Activation in 293T Cells

(A) 293T cells were cultured in DMEM-high glucose medium to maintain ATP supplies by glycolysis. Four hours after transfection with 9 μg of pcDNA-Bax (Bax) or control pcDNA (C) plasmids with 1 μg of pEGFP, the culture medium was exchanged with fresh medium containing or lacking 0, 0.1, 1, or 10 μM oligomycin or 10 μM antimycin A. After an additional 8 hr of culture, the cells were collected. In (A), the percentage of GFP^+ cells with apoptotic morphology was determined by DAPI-staining (mean ± SD; n = 3).

(B) 293T cells were recovered from cultures of untransfected cells and incubated with DiOC6, followed by FACS analysis. Data represent log fluorescence versus relative cell number. As a control for specificity of DiOC6 labeling, an aliquot of the control untreated cells was exposed to the protonophore mClCCP for 15 min prior to incubation with DiOC6.

(C) Lysates derived from cells that had been cultured with or without 10 μM of oligomycin were prepared and normalized for total protein content, and caspase activity was measured based on hydrolysis of DEVD-AFC (Deveraux et al., 1997). Typical substrate hydrolysis progress curves are shown (representative of three experiments).

(D) Aliquots of the same lysates employed for caspase assays were subjected to immunoblot analysis, employing anti-hu Bax antiserum with ECL-based detection. The blot was subsequently reprobed with anti-tubulin antibody to verify loading of equivalent amounts of total protein.

to pharmacological studies employing oligomycin. In mammalian cells, unlike in yeast, oligomycin is toxic and leads secondarily to mitochondrial PTP opening and either apoptosis or necrosis, depending on the particular cells and circumstances evaluated (Castedo et al., 1996). Cell death caused by oligomycin, however, can be delayed by culturing in high glucose–containing medium, which helps to maintain ATP levels via glycolysis (Eguchi et al., 1997; Leist et al., 1997). We therefore explored the effects of oligomycin in human 293T kidney epithelial cells grown in high glucose medium, using a transient Bax transfection assay to induce apoptosis and activation of cell death proteases (caspases) (Zha et al., 1996; Deveraux et al., 1997; Jürgensmeier et al., 1997). For all experiments, oligomycin was added 4 hr after transfections; caspase activity and apoptosis were then measured after an additional 8 hr of culture. Thus, the experiments were performed within the first 12 hr after Bax transfections, before oligomycin caused cell death (>90% trypan blue dye exclusion in control cultures).

As shown in Figure 5A, oligomycin reduced the percentage of apoptotic cells in cultures of Bax-transfected 293T cells in a dose-dependent manner, with concentrations of 1–10 μM oligomycin preventing approximately half of the Bax-induced cell death. $DiOC_6$-based measurement of mitochondrial membrane potential demonstrated that these concentrations of oligomycin resulted in hyperpolarization of mitochondria in 293T cells, consistent with a block of the proton pump causing accumulation of H^+ ions in the intermembrane space of these organelles (Figure 5B). Acute exposure of the cells to the protonophore mClCCP confirmed that the $DiOC_6$ staining was dependent on the mitochondrial pH gradient (Figure 5B), thus verifying the specificity of this assay.

In contrast to oligomycin, culturing Bax-transfected 293T cells with the respiratory complex III inhibitor antimycin A did not impair Bax-induced apoptosis under these conditions, but did markedly reduce mitochondrial $\Delta\Psi$ (Figure 5B). These findings in mammalian cells thus support the observations obtained with yeast, demonstrating again that respiration is unnecessary for Bax-mediated cell death.

Since gene transfer–mediated overexpression of Bax has been shown to induce activation of caspases that can cleave the substrate peptide DEVD (Deveraux et al., 1997; Jürgensmeier et al., 1997), we measured the effects of oligomycin treatment on Bax-induced activation of DEVD-cleaving caspases using lysates from the transfected 293T cells. As shown in Figure 5C, 293T cells transfected with pcDNA3-Bax contained markedly elevated levels of caspase activity compared to control transfected cells. Addition of 1–10 μM oligomycin to the cultures substantially reduced the amount of Bax-induced caspase activity (Figure 5C and data not shown).

Under these same conditions, ATP levels were maintained to within ~95% of control levels for 293T cells treated with 1 μM oligomycin (32 ± 3 nmol/mg protein versus 34 ± 3 nmol/mg protein) and to within ~75% of control levels for cells treated with 10 μM oligomycin (25 ± 2 nmol/mg protein). Thus, the oligomycin-mediated protection against Bax-induced apoptosis cannot be ascribed to reduced ATP levels. Oligomycin also did not impair production of the expected 21 kDa Bax protein in 293T cells (Figure 5D).

When used at high concentrations in vitro, oligomycin has been reported to inhibit the plasma membrane Na-K ATPase (IC_{50} ~ 5 μM) (Decottignies et al., 1995). We therefore tested the effect of the Na-K ATPase inhibitor oubain on Bax-induced apoptosis in 293T cells, but found that even at 100 μM, oubain had no influence on Bax function (not shown). Based on the above results,

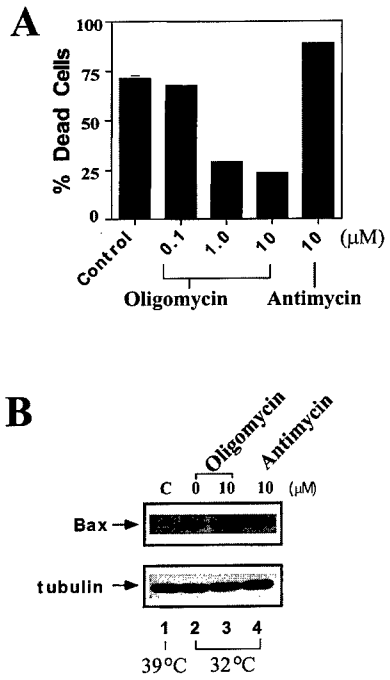

Figure 6. Oligomycin Inhibits p53-Induced Apoptosis

BRK cells that contain ts-p53 (Han et al., 1996) were maintained at a nonpermissive temperature of 39°C, then cultured at 32°C to induce p53 in the presence or absence of 0.1–10 μM oligomycin or 10 μM antimycin A. In (A), the percentage of dead cells was determined by DAPI staining 12 hr after shifting to 32°C (mean ± SD; n = 3). In (B), Lysates were prepared from BRK cells that had been cultured at 39°C (lane 1) as a control (C) or at 32°C (lanes 2–4) without or with 10 μM oligomycin or 10 μM antimycin for 12 hr, normalized for total protein content (5 μg/lane), and analyzed by immunoblotting using anti-Bax antiserum with ECL-based detection. The same blot was reprobe with anti-tubulin.

therefore, we conclude that the F_0F_1-ATPase proton pump is either required for optimal function of Bax in 293T cells or enhances Bax's ability to induce apoptosis in these human cells.

Oligomycin Inhibits p53-Induced Apoptosis

The induction of apoptosis in baby rat kidney (BRK) cells by p53 has been shown to be Bax-dependent (Han et al., 1996; Sabbatini et al., 1997). We therefore employed BRK cells that express a temperature-sensitive mutant of p53, and examined the impact of culturing these cells with various concentrations of oligomycin at either the permissive temperature of 32°C where p53 is active and apoptosis ensues or at the nonpermissive temperature of 37–39°C where p53 is inactive. As in the prior experiments, these cells were grown in high glucose medium to maintain ATP levels through glycolysis.

Oligomycin reduced the percentage of apoptotic cells by approximately two-thirds when ts-p53 BRK cells were cultured at 32°C to active p53 (Figure 6A). Under these same conditions, ATP levels were maintained at ~100% of control levels in BRK cells treated with 1 μM oligomycin and to within ~70% of control for 10 μM oligomycin (not shown). In contrast, antimycin A had no apparent effect on p53-induced apoptosis in BRK cells (Figure 6A), yet reduced ATP levels more than oligomycin. Immunoblot analysis of BRK cells demonstrated that neither oligomycin nor antimycin A (10 μM) impaired p53-induced expression of Bax when the cells were cultured at the permissive temperature of 32°C (Figure 6B). Taken together, therefore, these data provide further evidence that Bax-dependent apoptosis requires the mitochondrial F_0F_1-ATPase proton pump.

Discussion

Here we present genetic evidence that the mitochondrial F_0F_1-ATPase proton pump is required for Bax-induced cell death in yeast. By employing oligomycin, a specific inhibitor of the proton pump, we also found that mitochondrial F_0F_1-ATPase is apparently required for at least optimal induction of apoptosis and activation of caspases by Bax in mammalian cells. Based on currently available information, Bax and the F_0F_1-ATPase proton pump are thought to reside in different mitochondrial membranes, with Bax associated with the outer membranes oriented primarily toward the cytosol and the F_0F_1-ATPase in the inner membrane (Figure 7). Presumably, therefore, these proteins do not physically interact, though the tendency of Bcl-2 family proteins to concentrate at the junctional complexes of mitochondria, where the inner and outer membranes come into contact (Krajewski et al., 1993; de Jong et al., 1994), may create opportunities for a direct interaction. This might be particularly true when Bax is integrated into membranes within its capacity as a channel-forming protein, which is speculated to involve the insertion of the predicted 5th and 6th α helices of Bax through the lipid bilayer (reviewed in Reed, 1997).

Barring a direct physical interaction, how then might the F_0F_1-ATPase contribute to Bax-induced cell death? At least two potential explanations can be entertained. First, loss of the proton pump might prevent Bax from integrating into the outer membrane to form a channel. In this regard, the channels formed in vitro by Bax, as well as by Bcl-2 and Bcl-X_L, are voltage-dependent and their activities are modulated by pH (Antonsson et al., 1997; Minn et al., 1997; Schendel et al., 1997). For example, the conductances of Bax channels in planar bilayers are reportedly higher at neutral than at acidic pH, whereas Bcl-2 channels open more frequently at lower pH. In addition, most Bcl-2 family proteins including Bax contain a glutamic acid residue between the α5 and α6 helices. Protonation of this residue at lower pHs therefore could destabilize the membrane-inserted channel-forming conformation, allowing these two helices to more easily slip back out of the planar bilayer. Thus, alterations of the voltage potential across the inner mitochondrial membrane where the F_0F_1-ATPase resides or changes of pH at the surface of the inner membrane theoretically could interfere with Bax channel activity, assuming Bax can be influenced by the local voltage potential and pH gradient, particularly at the junctional complexes. The insertion of the α5 and α6 helices of Bax into membranes could also provide a way for exposing the BH3 domain of Bax so that the hydrophobic face of this α helix is available for dimerization with Bcl-2 family proteins (Figure 7), as revealed by recent structural studies (Sattler et al., 1997). BH3 domain–mediated dimerization could play an important role in

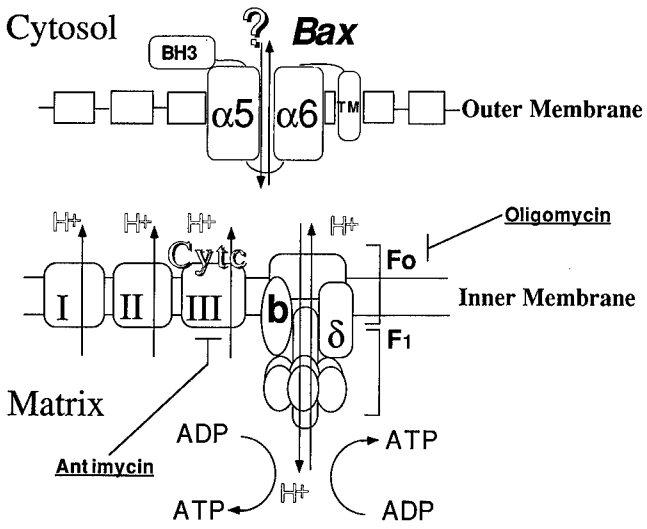

Figure 7. Schematic of Potential Relations between Mitochondrial F_0F_1-ATPase Proton Pump and Bax

The diagram depicts mitochondria, showing the inner and outer membranes. The outer membrane is thought to be porus because of porin. Bax in the outer membrane is anchored via a C-terminal transmembrane (TM) domain. During channel formation, the predicted 5th and 6th α helices of Bax are speculated to penetrate the lipid bilayer. The BH3 domain (α2 helix) is located on the cytosolic side of the membrane and can mediate dimerization with Bcl-2 and related proteins.

The F_0F_1-ATPase proton pump resides in the inner membrane. The β (equivalent to subunit 4 in yeast) and δ subunits are indicated. The β and δ subunits of F_0 and F_1, respectively, play important roles in connecting the F_0 proton channel and F_1 ATPase portions together. The transport of H^+ ions by the proton pump is reversible, and can either consume or generate ATP. Oligomycin shuts off the pump, such that protons cannot be transported in either direction.

The respiratory chain complexes I, II, and III extrude protons into the intermembrane space. Complex III is inhibited by antimycin A and associated with cytochrome c.

modulating interactions of Bcl-2 and Bcl-X_L with CED-4 or other proteins (Reed, 1997), irrespective of ion-channel formation, at least in mammalian cells.

A second possible explanation for why the F_0F_1-ATPase is required for Bax-mediated lethality in yeast is that the proton pump may be a downstream effector of Bax. In this regard, the F_0F_1-ATPase can operate in both forward and reverse directions, either transporting protons into the matrix down their concentration gradient and creating ATP, or pumping protons out of the matrix while consuming ATP (Figure 7). It is conceivable, therefore, that Bax channels render the outer membrane more porous, causing a faster dissipation of the proton gradient than usual through leakage of H^+ ions into the cytosol. As a secondary consequence, the F_0F_1-ATPase proton pump would be predicted to run in reverse, thus consuming ATP and alkalinizing the matrix by extruding protons. Since alkalinization of the matrix has been shown to cause opening of the mitochondria permeability transition (PT) pore in both mammalian and yeast mitochondria (Bernardi et al., 1994; Ichas et al., 1997; Jung et al., 1997), the F_0F_1-ATPase could theoretically facilitate Bax-induced cell death by this mechanism. In this regard, yeast mitochondria have been reported recently to have evidence of a PT pore that can be induced to open by matrix alkalinization (Jung et al., 1997).

In previous studies where oligomycin was used under conditions designed to maintain ATP levels and prevent necrosis, it was observed that cell death induced by etoposide and dexamethasone was inhibited (Eguchi et al., 1997; Leist et al., 1997). In contrast, apoptosis induced by anti-Fas antibody is apparently not dependent on the F_0F_1-ATPase, since Fas can still induce apoptosis in cells exposed to oligomycin in high-glucose media. Though this observation clearly suggests that the F_0F_1-ATPase is not absolutely necessary for apoptosis, it does not discount the possibility that the proton pump contributes to Bax-induced cell killing since Fas-induced apoptosis appears to be relatively Bax-independent (reviewed in Vaux and Strasser, 1996) whereas apoptosis induced by etoposide and dexamethasone can be assisted by Bax (Brady et al., 1996).

In summary, the data reported here demonstrate a role for the mitochondrial F_0F_1-ATPase in Bax-induced cell death. Though other explanations are possible, we suspect that the functional interaction between Bax and the proton pump is a manifestation of Bax's ability to function as a channel protein in mitochondrial membranes. However, Bax can promote cell death in mammalian cells by at least two mechanisms: (a) by forming channels in membranes; and (b) by dimerizing with anti-apoptotic Bcl-2 family proteins and thereby interfering with their actions independent of channel formation (Reed, 1997). In yeast, we hypothesize that the bioactivity of Bax is entirely dependent on its ability to form channels, since these simple unicellular organisms appear to lack Bcl-2 and CED-4 homologs. In mammalian cells, however, both mechanisms are likely to be operative. It remains to be determined which of these two mechanisms for Bax-mediated cell death (channel formation versus Bcl-2 antagonist) is quantitatively more important in mammalian cells, but cellular context and the specific cell death stimulus involved are likely to be highly important. Regardless, the data presented here suggest that applications of yeast genetics may provide novel insights into the channel-dependent mechanisms of Bax-induced cell death.

Experimental Procedures

Plasmids

YEp51-Bax, pEG202-Bax, and pcDNA-Bax have been described (Zha et al., 1996). The Bax cDNA from pEG202-Bax was subcloned

into the EcoRI and XhoI sites of pGilda (gift of C. Kaiser [MIT]). The YCp50 plasmid (ATCC37419) and yeast genomic library (ATCC37415) were obtained from American Type Culture Collection (Rockville, Maryland).

Yeast Methods

Yeast strains and plasmids used for these studies have been described previously (Velours et al., 1989; Paul et al., 1992; Giraud and Velours, 1994; Zha et al., 1996). For generation of Bax-resistant mutant yeast, EGY48 strain was mutagenized with MMNG (Sigma, Inc.) using routine methods (Guthrie and Fink, 1991). After treatment with MMNG for 10 min, yeast cells were grown for 4 hr in YPD media and then transformed by a LiOAc method with the *GAL10* promoter–containing plasmid YEp51-Bax and plated on minimal medium supplemented with required amino acid (MM-A) containing 1% raffinose and 2% galactose. Surviving clones were picked from plates and grown in MM-A with glucose prior to transformation with the *ADH1* promoter–containing plasmid pEG202-Bax (Sato et al., 1994) and selection on MM-A/2% glucose plates. Resistant mutants were mated with Myy290 strain (*Mata*, *his3*, *ura3*, *leu2*), and the resulting diploid cells were subjected to tetrad analysis (Guthrie and Fink, 1991). BRM1 cells displayed precisely 50% inheritance of the Bax-resistant phenotype.

Complementation Cloning

BRM1 cells was transformed with the *GAL1* promoter–containing plasmid pGilda-Bax and grown in MM-A with glucose to a density of 2–4 $\times$ 10^7 cells/ml. These cells were then transformed using a LiOAc method with 2 μg of a yeast genomic library in YCp50 (ATCC) and 20 μg of salmon sperm DNA (transformation efficiency 1 $\times$ 10^4 to 2 $\times$ 10^4 per μg DNA). Transformed cells were first plated on MM-A with glucose and then replica-plated to MM-A with galactose. From $\sim$2 $\times$ 10^4 independent colonies tested, 8 clones were identified that did not grow on MM-A/galactose plates. Plasmids were recovered from these 8 candidates, and BRM1 cells were retransformed with these library plasmid together with pGilda-Bax, thus confirming restoration of sensitivity to Bax to approximately the same level as wild-type yeast for 1 of them. The ends of this plasmid were sequenced by primers flanking the cloning site in YCp50 (5′-CGATCATGGCGACCA CACCCGTCCT-3′ and 5′-GGTGATGCCGGCCACGATGCGTCCG-3′). The DNA sequence results were compared with the Yeast Genomic Data Base using dbFAST (Stanford University).

Yeast Cell Viability Assays

Single colonies of yeast cells transformed with pGilda or pGilda-Bax were grown in 10–20 ml of MM-A/glucose with vigorous aeration at 30°C to an optical density of 0.4–0.5 OD_{600} nm. Cells were pelleted by centrifugation (1000 $\times$ g) for 10 min and washed three times in MM-A/galactose before resuspending in 20 ml of MM-A/galactose and culturing half with 10 μM oligomycin and half with ethanol solvent control (final 0.1 %). Oligomycin or ethanol was added to MM-A/galactose medium every 12 hr to ensure maintenance of adequate levels of drug. After culturing for various times at 30°C, a 0.5 ml aliquot of cells was removed for trypan blue dye exclusion assay, counting at least 300 total (live and dead) cells. Alternatively, the total cell density of cultures was determined, and either 1000 or 3000 cells were spread on MM-A/glucose plates, followed by culturing at 30°C for 4 days. The number of colonies on plates from the 0 hr cultures was designated as 100%.

Mammalian Cell Transfections and Apoptosis Assays

293T cells were cultured for 12 hr in DMEM-high glucose (4500 mg glucose/l) medium supplemented with 10% fetal bovine serum (FBS) at a density of 10^6 cells in 3.6 ml of medium per 6 cm diameter dish or 3 $\times$ 10^6 cells in 10 ml per 10 cm dish. Fresh medium was exchanged and 4 hr later the cells were transfected with 10 μg of pcDNA3-Bax versus parental pcDNA3 plasmid (10 cm dishes) or were cotransfected with 1 μg of pEGFP (Clontech Laboratories, Inc.) and either 9 μg of pcDNA3-Bax or pcDNA3 control plasmid (6 cm dishes). Four hours after the transfection, the medium was changed with fresh media containing 10 μM oligomycin, 10 μM antimycin A, or 0.1% ethanol (solvent). After culturing for an additional 8 hr, both the floating and attached cells were harvested. Half of the recovered cells were used for immunoblot assays and the remainder were used for either caspase activity assays (Deveraux et al., 1997) or for DAPI staining (Zha et al., 1996).

BRK cells expressing ts-p53 (Subramanian et al., 1995) were maintained in DMEM-high glucose 10% serum medium at the nonpermissive temperature of 39°C and cultured at either 5 $\times$ 10^4 cells/0.1 ml in 96-well flat bottom plates or at 10^6 cells/5 ml in 6 cm dishes for cell death assays. The medium was then changed with fresh 32°C medium, and cells were cultured at 32°C with or without various concentrations of oligomycin or antimycin A for 12 hr. The percentage of viable cells was determined by trypan blue dye exclusion, or cell lysates were prepared for immunoblot analysis of Bax expression.

Immunoblot Assays

Whole cell lysates were normalized for total protein content, and immunoblot assays were performed as described previously using 0.1% (v/v) anti-LexA rabbit serum (Zha et al., 1996) or either anti-human Bax or anti-mouse/rat Bax rabbit sera (Krajewski et al., 1994; Krajewski et al., 1995).

ATP Measurements

Cellular ATP content was measured as previously reported (Kane et al., 1985) using firefly lantern extract (Luciferase-luciferin, Sigma) with a luminometer. Data were normalized relative to total protein content of cell lysates.

Measurements of Mitochondrial $\Delta\Psi$

Mitochondrial $\Delta\Psi$ was measured using $DiOC_6$ (Molecular Probes, Inc.) as described (Castedo et al., 1996).

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